LATERAL INTERACTIONS IN THE PHOTORECEPTOR MEMBRANE

A NMR STUDY

L.C.P.J. Mollevanger

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Prof. Dr. C.W. Hilbers

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ABBREVIATIONS

CSA	Chemical shift anisotropy
Mops	Morpholino-propanesulfonic acid
NMR	Nuclear magnetic resonance
Pipes	1,4-piperazine-diethanesulfonic acid
PtdCho	Phosphatidylcholine
PtdEth	Phosphatidylethanolamine
PtdIno	Phosphatidylinositol
PtdSer	Phosphatidylserine

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Light is one of the most important media in nature by which an individual can obtain information about the environment. In animals this light information is captured by the eye. An image of the environment is projected, through a lens, on a light sensitive layer in the posterior of the eye. In this layer the incident light is converted into electrical signals that are transmitted to the brain.

The light sensitive layer in the posterior of the eye is an 0.1-0.5 mm thick cellular layer called the retina. In vertebrate retinas one can identify two types of light sensitive photoreceptor cells the rods and the cones. The names are derived from their morphological appearance [1,2]. The cone cells have a lower light sensitivity than rod cells. They function only under daylight conditions, and are responsible for color vision. This is facilitated by the fact that there are three types of cones each possessing a different pigment. The three pigments absorb light in the blue, green and red part of the spectrum with absorption maxima at respectively 440 , 535 and 575 nm [3].

The rod cells are much more sensitive to light than the cones. In fact one photon is sufficient to trigger the response of a rod cell [4]. The lowest level of light perception in the brain is 17 photons/s. The rod function is complementary to that of the cones. At low levels of light intensity light perception only occurs in the rod cells and is taken over by the cones at the higher light levels. In this way the eye is capable of detecting fluctuations in the light intensity over a total range of nine powers of ten. Since there is only one type of rod cell containing one type of pigment "rhodopsin", vision mediated by the rod cells is only in black and white ("grey values").

In most vertebrate retinas the number of rods outnumbers that of cones by a factor of at least seven [5]. Since in addition the rods are much easier to purify it is not surprising that the structure and function of rod cells is studied in much more detail then those of cones. This study will also only pertain to the rod cell, more specifically the bovine rod cell.

THE VERTEBRATE ROD CELL

A vertebrate rod cell can be divided in two different substructures, which are connected to each other through a fragile cilium (diameter 25 nm). Anterior located is the rod inner segment (RIM) containing the usual cell

organelles like endoplasmatic reticulum, Golgi complex, mitochondria, and nucleus. Most of the metabolic activity of the rod cell, like that involving energy supply and biosynthetic apparatus, takes place in the inner segment. It also has synaptic contacts with secondary neurons. These contacts are needed for the transduction of the light-induced electric signal.

The rod outer segment (ROS) is a highly specialized cell compartment responsible for the actual capture of the light photons. It has a cilindrical structure [6] , varying between the species in length (24 to 50 µm) and diameter(1.7 to 6 µm). When analysed by electron microscopy, it shows a regular stacked pile of 500 to 2000 flat membrane sacs called "disks" , which are surrounded by a plasma membrane. (Fig.1). The lamellar disk structure is highly regular, almost cristalline as shown by x-ray diffraction [7,8] with a disk to disk repeat distance of about 295 A [9,10]. The disks appear to be free floating without direct connections between the disks or to the surrounding plasma membrane. However there is substantial evidence for an interconnecting filamentous structure, linking the disk rims and outer segment plasma membrane [11,12].

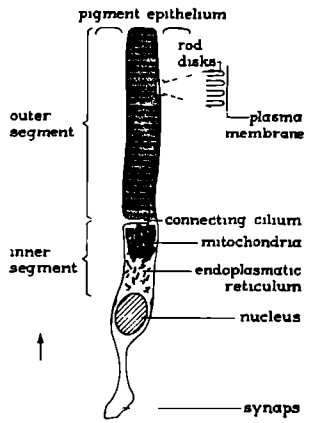

Figure 1 : Schematic drawing of a rod cell. The direction of the incident light is given by the arrow.

COMPOSITION OF THE ROD OUTER SEGMENT

The main components of the vertebrate rod outer segment are proteins and lipids. Intact non leaky rod outer segments contain nearly 30% water soluble and 70 % membrane bound protein [13]. Water washed rod outer segments consist

for 51 wt % of lipids (of which 81 wt % is phospholipid) and for 38 wt % of protein (of which > 90 wt % is rhodopsin) [14]. Rhodopsin is the predominant protein (over 90% on a protein base, ca. 35% on a total weight base) of the rod outer segment membrane. The remaining part of the total protein most likely represents membrane bound enzymatic activities [15].

THE STRUCTURE OF RHODOPSIN

In rod outer segment membranes rhodopsin is present in the monomeric form [16]. Rhodopsin consists of an apoprotein called opsin and a chromophoric group 11-cis retinal [1]. The spectrum of dark-isolated bovine rhodopsin shows three major absorption bands. The U.V. peak at 278 nm mainly represents aromatic amino-acid side chains. The main absorbance in the "visible region" is centered at 498 nm. This absorption arises in the chromophoric group. It enables rhodopsin to absorb light with a high efficiency (Molar absorption coefficient of 40.000 A.U. at 498 nm). The chromophoric group is bound to opsin by means of a Schiff base linkage, formed by the aldehyde of the chromophore and the amino group of Lysine-296, which is protonated by a protein residue [17].

Rhodopsin has a single polypeptide chain of 348 amino acids with a molecular weight of 39 KDa. The complete amino acid sequence was finally revealed in the early eighties by Ovchinnikov et al. [18] and Hargrave et al. [19] (Fig. 2). The amino acid sequence shows seven predominantly hydrophobic stretches varying in length between 19 and 28 residues. This suggests seven transmembrane segments in rhodopsin. Combining these data with those obtained by chemical modification and limited proteolysis, a model for rhodopsin has been proposed by Dratz and Hargrave [20] with seven trans membrane helices. An artistic drawing of this model is shown in (Fig. 3). Circular dichroism and Raman measurements indeed indicate as much as 60% α helical structure in rhodopsin [21]. The helices are largely oriented perpendicular to the plane of the membrane [10,22].

A more detailed description of the three dimensional structure can be obtained using the x-ray diffraction technique. It requires however good quality single crystals of rhodopsin. Up till now crystallization of a membrane protein like rhodopsin has proven to be extremely difficult and it has not yet been possible to obtain suitable single cristals [23,24].

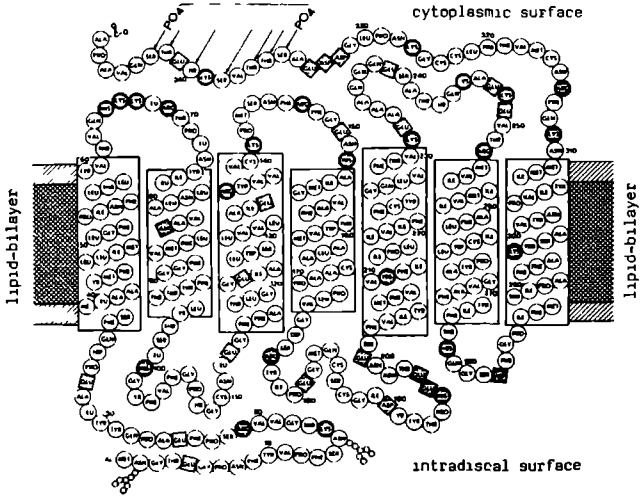

Figure 2 : A model for the organization of the polypeptide chain of rhodopsin in the photoreceptor membrane bilayer. Adapted from [20].

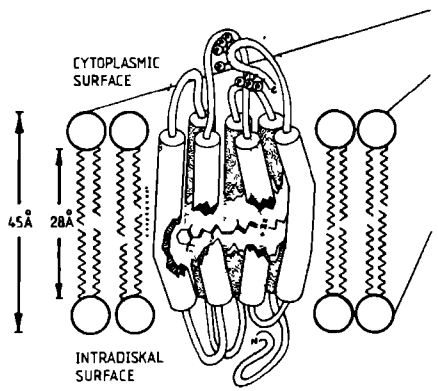

Figure 3 : Artistic drawing of the general structure of rhodopsin and its association with the photoreceptor membrane bilayer. The 11-cis retinal is oriented nearly parallel to the membrane plane. From [20].

The orientation of rhodopsin in the membrane is such that the carboxyl terminus is located at the cytoplasmic surface of the disk membrane and plasma membrane [25]. The amino terminus is at the at the interdiskal side of the disk membrane and at the extracellular side of the plasma membrane [26,27]. At the amino terminal end two carbohydrate chains, each consisting of six to seven monosaccharide units, are bound to the residues Asn-2 and Asn-15 [28].

1: THE PRIMARY REACTION IS A PHOTO-ISOMERIZATION

The primary event in vision is the absorption of a light photon by rhodopsin, in fact by the retinylidene chromophore of rhodopsin, causing an isomeric transition from the 11-cis to the all trans form. At room and physiological temperature a sequence of light independent "dark reactions" in the rhodopsin molecule takes place after the light induced isomerization ,which finally leads to the release of all-trans retinal from the protein [1]. (Fig. 4) shows the characteristic absorption spectra of rhodopsin before and after illumination which represent the two extreme states, rhodopsin and opsin + retinal. In the sequence of dark reactions a number of so-called photolytic

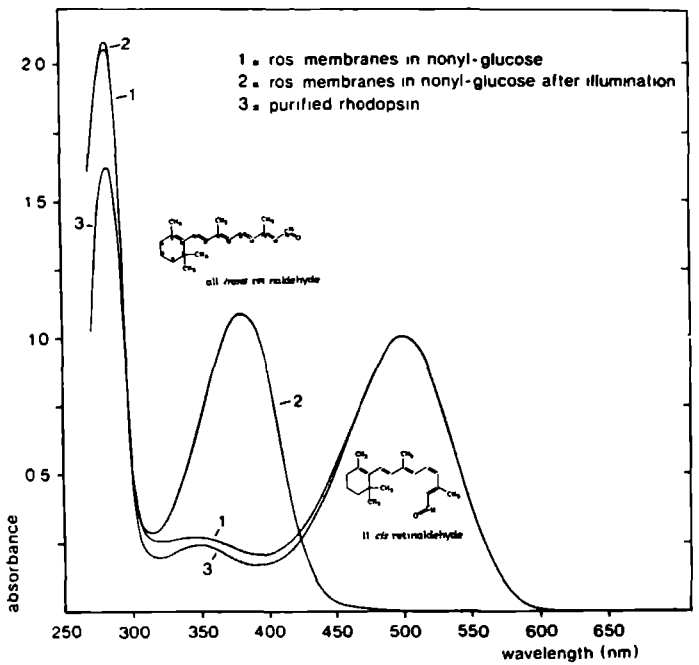

Figure 4 : Absorbance spectrum of washed, isolated rod outer segment membranes, solubilized in 20 mM nonylglucose, before (1) and after (2) illumination. (3) Purified rhodopsin before illumination.

intermediates can be distinguished on the basis of their spectroscopic properties [29]. Figure 5 lists the known rhodopsin photolytic intermediates and their lifetimes at room temperature.

All intermediates have been discovered by means of low temperature studies [1] and have been identified as genuine intermediates of the rhodopsin photocycle by flash photolysis at room temperature [30,31]. The photolytic cycle can be halted at a particular intermediate if rhodopsin is bleached in that particular temperature range. For a proper photolytic behaviour the presence of unsaturated lipids seems required, but already one double bond per acyl chain is sufficient [32]. This aspect will be dealt with in more detail later.

A light independent resensitation process in the retina allows the regeneration of rhodopsin from opsin and newly formed 11-cis retinal. In vitro this regeneration process can be mimicked by the addition of 11-cis retinal to opsin in the dark [33].

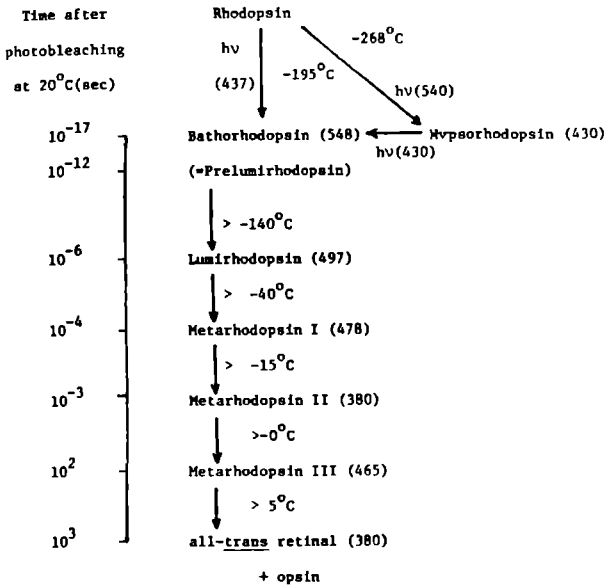

Figure 5 : Intermediates formed following photic bleaching of vertebrate rhodopsin. The absorption maximum of each intermediate is shown by the number of nanometers in parenthesis

The initial capture of a photon by rhodopsin is eventually expressed as a hyperpolarization of the plasma membrane of the rod outer segment. This process involves the following mechanism: In a dark adapted retina an extracellular current flows along the length of the rod cell [34]. Sodium ions are the primary charge carriers of this so called dark current. They enter the cell through the plasma membrane of the outer segment and leave the cell through the plasma membrane of the inner segment. The ionic gradients and electric balance necessary for the dark current are maintained by a Na^+/K^+ ion pump and a K^+ channel both located in the inner segment plasma membrane, and a Na^+-channel in the outer segment plasma membrane [35,36]. Illumination of the rod cell leads to a reduction of this current owing to a decrease in conductivity of the outer segment plasma membrane Na^+ channel. This causes hyperpolarization of the plasma membrane, to the K^+ equilibrium potential.

Since the disk membrane of the rod outer segment is electrically [37] and osmotically [8] isolated from the outer segment plasma membrane, an internal transmitter in the rod outer segment cytosol must be involved in coupling the bleaching of a rhodopsin molecule in the disk membrane to the decrease of the Na^+ permeability of the plasma membrane. In this process an amplification factor between 10^2 and 10^7 is achieved, meaning that the bleaching of one rhodopsin molecule blocks the influx of 10^2 to 10^7 Na^+ ions in the rod outer segment [38]. For this transducing amplification step two models have been proposed, one using Ca^{2+} ions [35] and the other using C-GMP [39,40] as the internal cytosolic transmitter.

The Calcium Hypothesis

The main features of the "Calcium transmitter model" are:

1) In the dark a low free Ca^{2+} concentration of approximately $0.5 * 10^{-6}$ M is maintained in the cytoplasmic space of the rod outer segment.
2) The Ca^{2+} concentration inside the disks is maintained at a much higher level than in the outer segment cytoplasmic space.
3) Upon absorption of light by rhodopsin Ca^{2+} is released from the disks and the cytoplasmic free Ca^{2+} concentration is increased, causing the Na^+ channels to close.
4) Ca^{2+} extrusion across the plasma membrane and re-uptake across the disk membrane is required to remove the released Ca^{2+} from the cytoplasma

re-establishing the original conditions.

The Ca^{2+} hypothesis is supported by experimental evidence showing that: A) The dark current is strongly influenced by the extracellular Ca^{2+} concentration [41,42,43] , B) Lowering of the intracellular free Ca^{2+} concentration in rods increases the membrane conductivity for Na^{+} ions [44]. There is however still no solid evidence for the release of Ca^{2+} ions from the rod disks in sufficient amounts and with a rate satisfying the Ca^{2+} hypothesis. Also the process of the active accumulation of Ca^{2+} ions into the rod disks is still unclarified.

The Cyclic GMP Hypothesis

The essential features of the cyclic GMP hypothesis are:

1) After the absorption of a photon rhodopsin undergoes a number of conformational changes. The meta rhodopsin II is the active intermediate, which binds and in turn activates up to 500 G-protein molecules to exchange GDP for GTP per second.
2) The GTP G-protein complex activates a phophodiesterase to hydrolize about 200 cGMP molecules/sec/G-protein.
3) The Na^{+}-conductance of the rod outer segment plasma membrane is lowered by a reversible dephosphorylation of a protein of the Na^{+}-channel, caused by the drop in the cGMP concentration in the cytosol.

There are several strong arguments supporting the direct involvement of cGMP in photoregulation of the Na^{+}-permeability of the rod outer segment plasma membrane: A) In isolated rod outer segments the level of cGMP is rapidly (halftime of 100-200 msec) reduced by illumination by as much as 50-70% [45,46], B) there is a light intensity dependent metabolic turnover of cGMP in the intact retina [46,47], C) Microinjection of cGMP into the rod cell is usually accompanied by depolarization of the rod, and a decrase in its sensitivity for light [49]. Still unresolved however is the precise molecular mechanism of step 3 of the cGMP hypothesis. A clear correlation between phosphorylation reactions and photoresponse has not yet been demonstrated. Recent experiments indicate that cGMP might very well interact directly with the Na^{+} channels [50]. This would mean that the principles of the entire photo excitatory pipeline, from the photoisomerization of retinal to the closure of the Na^{+} channels [49], have been clarified. confirmed.

What about the Ca^{2+} hypothesis? The release of Ca^{2+} ions from the disks seems to be controlled by a sequence of reactions that uses the same

initial GTP G-protein cascade as the cGMP pathway. Intracellular Ca^{2+} can definitely modulate the Na^{+} channel conductance, the response to light however being slower and less sensitive than that of the cGMP pathway. Ca^{2+} ions have probably a major role in adapting the cell to constant illumination (light-adaptation) in order to increase its dynamic range.

LIPID COMPOSITION OF ROD OUTER SEGMENT MEMBRANES

The lipid species and the number of lipid molecules per rhodopsin molecule have been analysed in detail [51,52,53,54]. There are about 65 phospholipids, 2 diglycerids, 4-5 free fatty acids, 1 retinal, and 5-8 cholesterol molecules per rhodopsin molecule in bovine rod outer segments.

The phospholipid headgroup classes found in the rod outer segment membrane are phosphatidylethanolamine (PtdEth), phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIno), and sphingomyeline (SPH), of which the former three are by far the most abundant ones (Table.1). Analysis of the fatty acid composition of the phospholipids reveals a very high content, up to 55%, of polyunsaturated fatty acids, with an average number of 5.9 double bonds per phospholipid. The most abundant unsaturated fatty acid ,docosahexaenoic acid (22:6), comprises about 40% of the total phospholipid fatty acid pool. The saturated fatty acids mainly consist of palmitic acid (16:0) and stearic acid (18:0), ca. 21%.

Table 1 : Phospholipid composition of the photoreceptor membrane expressed as mole phospholipid/mole rhodopsin.

Phospholipids	mole/ mole rhodopsin
Phosphatidylethanolamine	31
Phosphatidylcholine	24
Phosphatidylserine	10
Phosphatidylinositol	0.7
Sphingomyeline	0.6

Analysis of the fatty acid composition of the individual phospholipids classes shows that phosphatidylethanolamine and phosphatidylserine are enriched in docosahexaenoic acid (22:6) relative to phosphatidylcholine. While

these three phospholipids have roughly the same content of stearic acid (18:0), their content of palmitic acid (16:0) increases in the order phosphatidylserine < phosphatidylethanolamine < phosphatidylcholine. Phosphatidylserine further contains appreciable amounts of relatively long chain fatty acids (24:4-24:6). The high content of polyunsaturated fatty acids should give the photoreceptor membrane matrix special properties. Although it has been suggested that this would make the membrane lipid matrix very fluid, the rate of rotational movement [55,56] and translational diffusion [57] of rhodopsin in the plane of the membrane indicate a membrane viscosity in the range of 1-10 poise, which is not exceptionally fluid compared to other membranes. The photoreceptor membrane exhibits a gel to liquid phase transition around -20 ^{o}C indicating that at physiological temperature the membranes are in the liquid crystalline state [58].

This high degree of unsaturation appears to be essential for proper functioning of the rod photoreceptor cells. Rats kept on a diet depleted of the essential fatty acids linoleic (18:2) and linolenic (18:3) acid, precursors for 22:5 and 22:6 respectively, show a rapid depletion of most tissues, except for retina, which tenaciously holds to its level of unsaturation. Reduction of this level seems to impair the visual transduction process [59].

The very high content of poly-unsaturated fatty acids can have a considerable influence on the lipid phase behaviour including bilayer to hexagonal H_{II} phase transitions. This aspect will be discussed in the next section.

MEMBRANE STRUCTURE

The fluid mosaic model.

The basic structure of biological membranes is currently presented in the fluid mosaic model as first presented by Singer and Nicolson [60]. In this model the lipids are thought to be arranged in a bilayer with the hydrophylic moieties exposed to the aqueous medium and the hydrophobic hydrocarbon chains of both the molecular monolayers (inner and outer) joining at the center of the bilayer. This provides a semipermeable barrier, and separates the cell or organelle contents from their surroundings. In addition the lipid bilayer forms a structural backbone and acts as a matrix for membrane proteins, which carry the selective permeability of the membrane. Membrane proteins can

roughly be divided into two catagories, intrinsic membrane proteins which either span the membrane or are partially embedded in it and the extrinsic membrane proteins which are only associated with the inner or outer monolayer. The idea that the lipid matrix, and not the membrane protein,is responsible for the basic structure of biomembranes is supported by the observation that many natural and synthethic phospholipids spontanuously adopt a bilayer configuration upon hydration [61].

The lipids found in biological membranes show a great diversity with respect to polar headgroups, fatty acid composition, and occurrence of sterols. Thinking of a biological membrane in terms of the fluid mosaic model, this diversity may serve to control protein function by providing regions of different fluidity or locally different composition. It is quite possible that different degrees of molecular packing and mobility occur in different regions of the membrane.

It seems however not very likely that the gel to liquid crystalline transition can serve as a general mechanism for the regulation of membrane functions, because the lipid matrix in most biological membranes and particularly in mammalian is in a liquid crystalline state [62]. In addition, metabolic active membranes in which regulation of protein activity by lipid fluidity would be expected to be most pronounced, appear to contain high amounts of polyunsaturated fatty acid chains and consequently will not easily go into the gel state [63].

Based on early ESR, NMR and biochemical experiments a long living immobilized lipid boundary around membrane proteins has been proposed, to regulate protein activity [64] , However the exchange of lipid molecules at the protein surface occurs on a very short timescale, ca. 10^{-4} sec, compared to the turnover number of most enzymatic reactions [65,66]. Therefore the annular lipidmatrix as such is not to be expected essential for the functioning of membrane enzymes. It seems in this respect to be more appropiate to look at this in the reverse way. Proteins could influence the local order and dynamics of the membrane lipids.

Nevertheless, some enzymes seem to depend on the presence of special lipids in the membrane to facilitate a proper function of the enzyme. For example cytochrome-C oxidase has an absolute requirement for cardiolipin. But this requirement is already satisfied at 3-4 cardiolipin molecules per cytochrome oxidase, which hardly can be considered a lipid annulus [67].

Although in a limited number of cases a special lipid composition of the

membrane is required for the correct functioning of a specific enzyme, in general this cannot satisfactorily explain the wide variety of lipid components, and lipid compositions found in biological membranes. Especially if one considers that in terms of the fluid mosaic model for the lipid matrix the properties of a biological membrane could very well be sustained, replacing all lipids by one type of unsaturated phosphatidylcholine. The fluid mosaic model only allows the lipids to be organized in a perfect bilayer with fluidity and lateral distribution as the sole dynamic parameters. However a number of processes occurring in biological membranes such as membrane fusion, exo- and endo- cytosis, ion conductivity, protein insertion and transbilayer movement of lipids are difficult to imagine when the lipids are only allowed to have a bilayer organization.

Lipid polymorphism

In the last decade it has become clear that the structural view of the fluid mosaic model of lipids in a membrane is too simple. In particular the notion that each biological membrane contains substantial amounts of lipids which upon isolation and dispersion in aqueous buffers do rather organize themselves in non-bilayer configurations has suggested that lipids might play additional structural and functional roles [68]. The most commonly encountered non bilayer configurations adopted by lipids in model membrane systems are the hexagonal H_{II} phase cilinders and the related lipidic particles (inverted micelles). The idea that bilayer organized lipids in a membrane may reorganize into non-bilayer structures, which create exchange and perturbation sites in the membrane, has led to the concept that the role of membrane lipids is not merely to constitute a permeability barrier, but that they are involved in a variety of structural and functional aspects of biological membranes, including trans bilayer transport, lipid flip-flop, membrane fusion, and protein insertion [68].

Unambiguous proof for the existence of nonbilayer lipid structures in biological membranes under physiological conditions is still lacking. However there are now several morphological and functional indications which suggest that those structures can indeed occur transiently, or under special conditions, in a number of membranes [69,70,71,72]. From a functional viewpoint it is clear that mechanisms should exist by which the structure of lipids can be locally and/or transiently modulated to allow specific processes to occur at discrete sites. Special lipid-protein interactions seem to be the

most likely candidates to exert such a regulatory function.

LIPID POLYMORPHISM AND MOLECULAR SHAPE

In the previous sections it has been put forward that hydrated phospholipids have the ability to organize in different macroscopic structures depending on the environmental conditions. While studying the polymorphic phase behaviour of many phospholipids under various conditions Cullis and de Kruijff and coworkers [68,72,73,74], derived a simple rational between the overall molecular shape of a phospholipid and its phase behaviour. They developed a model based on the ratio between the cross sectional area of 1) the polar headgroup and 2) the hydrophobic acyl chain of a phospholipid. In general this model states that in aqueous dispersions (excess water) under physiological conditions of ionic strength and pH the following relation between molecular shape and the organization in lipids can be deduced:

(A) The inverted cone shape : Cross sectional area of the hydrophylic headgroup is larger than the area occupied by the hydrophobic moiety. It leads to the formation of micellar organization. Lipids : lysophospholipids and fatty acids

(B) The cilindrical shape : Both cross sectional areas do not differ very much. Phospholipids having this molecular shape easily adopt the bilayer organization. Lipids : PtdCho, PtdSer, SPH, PtdIno.

(C) The cone shape : Cross sectional area of the hydrophylic headgroup is smaller than the area of the hydrophobic acyl moiety. Phospholipids with this molecular shape prefer to adopt the hexagonal H_{II} phase. Lipids : PtdEth, $PA\text{-}CA^{2+}$.

Using the molecular shape model it is possible to understand and in some cases predict the polymorphic phase behaviour of many phospholipids. For instance the fact that unsaturated phosphotidyl ethanolamine in aqueous dispersions at neutral pH readily organizes in a hexagonal H_{II} phase, in contrast to phosphatidyl choline which strongly prefers the bilayer phase. It can now be rationalized on basis of the small ethanolamine headgroup in comparison to the hydrated bulky choline headgroup. Also the observation that the temperature of the bilayer to hexagonal H_{II} phase transition of unsaturated phosphatidylethanolamines decreases with increasing unsaturation in the acyl chain fits nicely in the molecular shape model. The area occupied by the hydrophobic acyl chain increases when going from a fully saturated to a

highly unsaturated acyl chain.

The induction of the bilayer to hexagonal H_{II} phase transition in lipid dispersions containing cardiolipin, phosphatidic acid or phosphatidyl ethanolamine by divalent cations (e.g. Ca^{2+} ions) is most probably caused by a reduction of hydration of the headgroup of these phospholipids upon binding to divalent cations. As a consequence the effective cross sectional area of the headgroup is again reduced, leading to a cone shaped molecular form.

This molecular shape model, in which the phospholipids are divided in three classes, is of course an oversimplification. However it has proven to be a very useful concept in the understanding of the molecular aspects leading to lipid polymorphism.

INFLUENCE OF MEMBRANE PROTEINS AND POLYPEPTIDES ON PHOSPHOLIPID POLYMORPHISM

By now it is well documented that phospholipids are capable of forming non-bilayer structures in aqueous dispersions. [74]. The occurrence of non bilayer structures depends, apart from the phospholipid composition, on pH, ionic strength, and temperature. Additional to these parameters, the phase behaviour of phospholipids can also be modulated by membrane proteins (both extrinsic and intrinsic) and some polypeptides [75]. Cytochrome-C, Cardiotoxin or Gramicidine were shown to stimulated the formation of non-bilayer phase in model membranes. On the other hand, intrinsic membrane proteins like Glycophorin, Ca^{2+} ATPase and Chlorophylase seem to stabilize the bilayer phase of phospholipids [75].

The nature of the lipid-protein interactions that allow membrane proteins to influence lipid polymorphism are not well understood. However a few mechanisms have been suggested [75]. For a protein which promotes formation of the hexagonal H_{II} phase, like Gramicidine, several arguments have been put forward; 1) dehydration of the phospholipid headgroup, 2) decrease of the acyl chain order thereby decreasing the area occupied by the acyl chain moiety, both an effect of protein-lipid interaction and 3) Cone shape of the protein molecule itself. For a protein which stabilizes the bilayer phase like Glycophorin the following arguments are suggested: A) a large charged hydrophylic headgroup of the protein could prevent interbilayer fusion, an event which preceeds the formation of the hexagonal H_{II}, B) a large protein headgroup cannot fit into the narrow aqeous channel present in

the hexagonal H_{II} phase, C) the membrane spanning part of the protein possesses a large amount of α-helix giving the protein an overall cilindrical shape, have been suggested.

TECHNIQUES TO STUDY LIPID PHASE BEHAVIOUR

There are essentially three different techniques to study the configuration of hydrated lipids in aqueous suspensions:

Small angle x-ray diffraction.

This technique has the advantage that it provides an unambiguous phase determination. A disadvantage is the long exposure times and its requirement of long range order in the sample. Therefore no detailed information can be obtained from small structures like sonicated vesicles which are randomly ordered. Furthermore when different lipid configurations are present within one sample, it is difficult to determine presence or quantity of the less predominant phase. This technique is therefore limited in its use and not generally applicable in the study of biological membranes. For a review see [76].

Electron microscopy

Freeze fracture electron microscopy has more potentials as a technique for the study of lipid polymorphism in membranes. First of all as a microscopical technique it is capable to detect different lipid configurations present simultaniously within one sample. Such in contrast to x-ray diffraction and calorimetric techniques which only give an average signal from a sample. However it is difficult to obtain reliable quantitative information on different coexisting lipid phases. Unfortunately technical artifacts, like reorientation of lipid during cooling, remains a possible source of error in particular with lipids that undergo low energy transitions such as a hexagonal H_{II} phase to bilayer transition [77].

^{31}P-NMR

A third approach for the study of lipid polymorphism, which developed in the past 10 years to a very powerful technique in this field, is ^{31}P-NMR spectroscopy. Indeed this technique has important advantages. First, it allows a convenient and quantitative discrimination of most coexisting lipid phases in aqueous dispersion. Secondly it gives information on dynamic properties of lipids like translational and rotational freedom, order parameters etc. Thirdly, samples can be directly studied at physiological conditions, in

aqueous dispersions, without the need for extra manipulation. Therefore changes induced by parameters like pH, cations, or temperature can be directly observed. Such potential makes the ^{31}P-NMR technique generally applicable to study lipid polymorphism in model membranes and biological membranes [68,78,79].

Because the larger part of the results presented in this thesis rely on ^{31}P-NMR spectroscopy, a short theoretical description of the relation between the lineshape of a ^{31}P-NMR spectrum and the corresponding organization of the phospholipids in a membrane suspension will be given.

Almost all phospholipids contain only one phosphorous atom per molecule, in the form of a phosphate group. Here the phosphorous atom is asymmetrically surrounded by 4 oxygen atoms. The electron density distribution around its nucleus is therefore anisotropic. As a result the local magnetic shielding, and thus the chemical shift of the resonance from a phosphate group depends on its relative orientation to the B_o magnetic field. This anisotropy of the chemical shift can be described by a static axially asymmetric tensor.

$$\sigma_p = (\sigma_{11}, \sigma_{22}, \sigma_{33})$$

For phospholipids the principal chemical shielding tensor elements can easily be obtained from dry powder proton decoupled ^{31}P NMR spectra. Typical values for σ_{11}, σ_{22} and σ_{33} are -80 ppm, -20 ppm and +110 ppm [79]. ^{31}P NMR spectra of hydrated phospholipids however show lineshapes that are consistent with an "axially symmetric" chemical shielding tensor. The originally 3 tensor elements are then reduced to 2, called $\sigma_\perp$ and $\sigma_{||}$. This reduction in the number of tensor elements is caused by the fast rotation (on the NMR timescale) of the phosphate segment of phopholipids around their long axis (parallel to the bilayer normal) which occurs in fully hydrated condition and which averages the contributions of σ and σ that lie in the plane of the membrane. [78]. The spectral width of a ^{31}P NMR spectrum is now defined by :

$$\delta\sigma = \sigma_\perp - \sigma_{||}$$

And the isotropic chemical shift by :

$$\sigma_i = \frac{1}{3}(\sigma_{11}+\sigma_{22}+\sigma_{33}) = \frac{1}{3}(\sigma_{||}+2*\sigma_\perp)$$

Apart from this fast rotation a phospholipid molecule in a large lipid structure like a bilayer shows only slow translational or vectorial movements on the NMR timescale. Therefore the lineshape of ^{31}P-NMR spectra taken from phospholipid dispersions directly reflects the angle dependent intensity distribution of the phospholipids in the sample. It can now be shown by simple calculation that the lineshape of the ^{31}P-NMR spectrum shown in figure 6A, represents lipids in a bilayer arrangement in large round vesicular structures ($\varnothing$ > 200 nm) [78]. Typical characteristics are the high field peak, low field shoulder and a chemical shift anisotropy of approximately 40 to 50 ppm. In the Hexagonal H_{II} phase, with its long tubular structures, the angle dependent intensity distribution of the phospholipids is 90^{o} rotated compared to that found in the previously discribed bilayer. Also the rotational freedom of the lipids in the H_{II} phase is enhanced compared to lipids in a bilayer structure. Considering this, it is easy to show that phospholipids organized in the Hexagonal H_{II} phase yield ^{31}P-NMR spectra with a high field peak and a low field shoulder and a chemical shift anisotropy $\Delta\sigma$ of approximately 25 ppm (Fig. 6B).

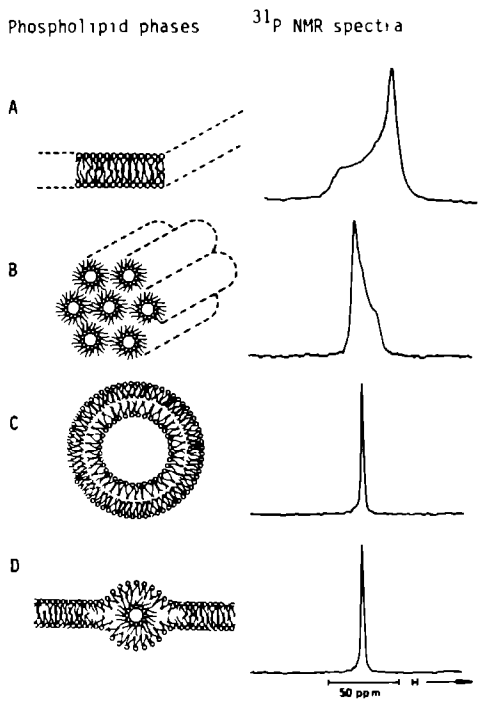

Figure 6 : ^{31}P-NMR spectra of phospholipids in various phases: (A) Bilayer phase, (B) Hexagonal H_{II} phase. Phases which allow fast isotropic motion (C) Sonicated vesicles and (D) Inverted micelles.

Comparison of experimental data on lipid polymorphism obtained by ^{31}P-NMR spectroscopy with those obtained by freeze fracture electron microscopy or x-ray diffraction, as published by Cullis and de Kruijff and coworkers during the last ten years [68,74,75] clearly shows that there is an unambiguous relation between the occurrence of the above described lineshapes in a ^{31}P NMR spectrum and the presence of a certain phospholipid organization. It has been noted by Thayer and Kohler [80] that in theory a change only in the headgroup structure of phospholipids organized in a bilayer can generate lineshapes in a ^{31}P NMR spectrum that are typical for phospholipids organized in the hexagonal H_{II} phase or phospholipids experiencing fast isotropic reorientation. However the phosphate segment of a phospholipid headgroup has a stable structure and therefore their suggestions remain speculative in the absence of experimental evidence [75]. Therefore apart from these theoretical considerations it is now generally accepted that the lineshape of a ^{31}P-NMR spectrum provides unequivocal evidence for phospholipids organized either in an extended bilayer or in a hexagonal H_{II} phase.

When phospholipid molecules are allowed fast isotropic reorientation (on the NMR timescale, $\tau_c < 10^{-5}$ sec); as occurs in very small vesicles ($\emptyset$<100 nm), micelles or inverted micelles (the so called "lipidic particles") the ^{31}P NMR spectrum shows motional narrowing to an isotropic line centered around σ_1. Hence, in the case of an isotropic line in the ^{31}P NMR spectrum additional evidence from e.g. freeze fracture electron microscopy is needed to unambiguously determine the conformation of the phospholipids in the sample (Fig 6C,D).

AIM OF THIS STUDY

The photoreceptor membrane from vertebrate rod outer segments represents an attractive model for the study of lipid protein interactions in native biological membranes because (A) rhodopsin comprises > 95 wt% of the intrinsic rod outer segment membrane protein, (B) rhodopsin spans the membrane and is present in high density. It should therefore have a large contact area with the membrane lipidmatrix, (C) the rod outer segment phospholipid composition is well characterized. It has the highest content of polyunsaturated fatty acyl chains found in any biological tissue up till now. This is particularly interesting because the degree of unsaturation of the acyl chain strongly

influences the polymorphic phase behaviour of phospholipids [74,75,81,82], (D) the photochemical behaviour of rhodopsin has been well studied and at least some aspects appear subject to modulation by the lipid environment, (E) rod outer segments can be easily isolated in high yield from bovine eyes obtained at the local slaughterhouse [14].

From the previous sections it is clear that the model of Singer and Nicholson [60] is too simple a concept of the biological membrane. The lipid bilayer model cannot explain important biological processes like membrane fusion, protein insertion and lipid flip-flop. It became clear in the early 1970 ties from the study of lipid phase behaviour in artificial model membranes that lipids are capable of adopting non bilayer organization, which can explain for the above mentioned phenomena. However up till now unambiguous proof for the existence of non-bilayer lipid structures in a biological membrane is still lacking [74,75,83].

The photoreceptor membrane has an exceptionally high content of polyunsaturated fatty acyl chains combined with a high amount of phosphatidyl ethanolamine. It is situated in a cell organelle, the rod outer segment, with a high biological activity in which controlable trans-membrane currents of different ions play an important role. These characteristics make it a very interesting biological membrane to search for the existence of non-bilayer structures. From the point of membrane biology as the first biological membrane that might be capable of producing detectable amounts of lipids organized in non-bilayer phases. From the field of vision since the existence of non-bilayer structures, especially when induced by a light dependent stimulus, could give a more detailed insight in the processes occurring in the disk membrane after absorption of a photon, which are the primary processes in vision that eventually trigger the hyper polarization of the plasma membrane resulting in an electric stimulus to the brain.

Therefore in this thesis a detailed study of the polymorphic phase behaviour of the rod outer segment photoreptor lipids was undertaken, concerning modulation of the polymorphic phase behaviour of photoreceptor membrane lipids by divalent cations and temperature (Chapter 3), polymorphism of the individual phospholipid classes phosphatidylethanolamine and phosphatidylserine and effects of cholesterol (Chapter 4), bilayer stabilization by (rhod)opsin (Chapter 5). Morphologically intact rod outer segment posseses a large magnetic anisotropy. This property is used to obtain ^{31}P-NMR of oriented photoreceptor membranes which allows spectral analysis

and identification of individual phospholipid classes (Chapter 6), and allows to study lateral lipid diffusion in intact disk membranes (Chapter 7).

An interesting sideline study, not dealing with lipid polymorphism is described in Chapter 8. When 11-cis retinal is bound to opsin forming rhodopsin a large red-shift is observed. Conformational changes forced upon the chromophore and interaction with negative charges of the apo-protein are responsible for this shift. The exact nature of interactions between the retinal chromophore an the protein moiety in rhodopsin is still unclear. The power of high resolution solid state ^{13}C-NMR to study the conformation of the chromophore in rhodopsin is demonstrated.

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CHAPTER 2

ANALYSIS AND ISOLATION OF PHOSPHOLIPID CLASSES FROM BOVINE ROD OUTER SEGMENTS BY HPLC AND GLC : A NEW SERIES OF VERY LONG CHAIN FATTY ACIDS.

Vertebrate photoreceptor membranes contain phospholipids with an extremely high percentage (over 55%) of poly-unsaturated acyl chains. Such a high degree of unsaturation will influence the physico chemical properties of membranes [1]. Furthermore, the three main phospholipid headgroup classes (phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine) considerably differ in their degree of unsaturation [2,3]. More insight in the contribution of the phospholipid classes to the overall properties of the photoreceptor membrane can only be obtained through examination of the isolated phospholipid classes. Hence, a rapid and reliable procedure to purify the phospholipids from photoreceptor membranes is desirable. Published methods for the isolation of phospholipid classes from photoreceptor membranes use two-dimensional TLC for purification on an analitical scale and conventional silicic acid column chromatography for isolation on a preparative scale [2,3]. Both methods are laborious and time consuming. Special precautions have to be taken to prevent oxidation of the poly-unsaturated fatty acyl chains (addition of anti-oxidants, elution under an inert atmosphere). Here we describe a simple and rapid procedure for the purification of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine from photoreceptor membranes on both an analytical and preparative scale, based on straight-phase gradient HPLC. The procedure is based on the approach described by Nissen et al. [4] for the analysis of phospholipids from human semen.

Recently Aveldano et al. [5] showed that ultra-long fatty acyl chains (C_{28} to C_{36}) were incorporated in phospholipids of bovine photoreceptor membranes, in particular in phosphatidylcholine. The distribution of these ultra-long fatty acids over the phospholipid classes obtained by our HPLC-procedure was analysed. Since phosphatidylcholine elutes in four subpeaks these were separately investigated. It appeared that the species with ultra-long fatty acids could be particularly purified in this way. Furthermore, the materials and methods section of this chapter presents a more detailed description of the isolation of photoreceptor membranes so that in the later chapters a brief description will suffice.

Isolation of photoreceptor membranes

Principle:

Rod outer segments are stacked full with photoreceptor membranes (over 70% of dry weight). Isolation of the latter is most conveniently done by first isolating intact rod outer segments, since their isolation is facilitated by two facts. First, rod outer segments are connected to the main cell body of the rod cell by a thin connecting cilium only, and they break off easily upon mild homogenization of the retina. Secondly, their high membrane content added to the high lipid content of the photoreceptor membrane results in a relatively low density (about 1.11-1.12), which permits separation from cells and other cell organelles by means of density centrifugation.

Rod outer segments are isolated from bovine retinas in a four step procedure. 1) Dissection of the eye and collection of the retina. 2) Homogenization of the retinas to break of the rod outer segments. 3) Separation of a crude rod outer segment fraction from larger fragments. 4) Purification of rod outer segments from contaminating cell organelles and membrane material by means of density centrifugation.

General procedure:

All operations are carried out in dim red light (λ < 620). Only fresh non-frozen retinas are used.

1. Bovine eyes are collected at the slaughterhouse in a light-tight container and dissected within 2 hours after the death of the animal. The retinas are removed and transferred to ice-cold isotonic isolation buffer in a Potter-Elvehjem tube (0.5 ml/retina of a solution of 20 mM Mops, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 140 mM NaCl, pH=7.2 and 1 mM DTE added just before use).

2. The retinas are mildly homoginated by 10 strokes with a loosely fitting Teflon pestle.

3. The homoginate is filtered through a nylon screen (125 mesh) by gentle swirling to remove larger tissue debris.

4. The filtrate is layered upon a sucrose density gradient (23% to 36% w/w sucrose in three times diluted isolation buffer) and subsequently centrifuged (80,000xg, 10° C, for at least 1.5 hour). After centrifugation a thick turbid band is observed in the middle of the gradient, which nearly representing almost pure rod outer segments. This band is carefully removed with a poly-ethylene syringe. To remove small contaminating membrane fragments

and vesicles, the rod outer segment suspension is carefully diluted with one volume of isolation buffer, and the rod outer segments sedimented by low speed centrifugation (2000xg, 5 °C, 10 min).

The resulting rod outer segment pellet can be stored under Argon at -70° C for at least a year. The yield is 30-50%, based on the total rhodopsin content of the retina. A measure for the purity of the isolated material is the ratio of its absorptions at 500 and 280 nm (A_{500}/A_{280}). Typical values for pure preparations vary between 2.3-2.5. For further details see [6]. A hypotonic lysis and washing step to remove cytosolic proteins results in pure photoreceptor membranes, which yields typical A_{500}/A_{280} ratio's of 1.9-2.1.

Preparation of total lipid extracts

Total lipid extracts were obtained from water-washed photoreceptor membranes by a modified Folch procedure [3]. One volume of membrane suspension is mixed with twenty volumes of CH_2Cl_2/MeOH (2/1 ; v/v) in a glass stoppered round bottem tube. Extraction is performed by mechanically shaking the mixture for 30 min (Griffin flask shaker, room temperature). Upon subsequent centrifugation (2500 rpm, 10 min) a supernatant and pellet are obtained. The pellet is reextracted with the same volume of CH_2Cl_2/MeOH, now containing 0.25% of a concentrated HCl solution. The combined supernatants are washed by hand-mixing with 0.2 vol. 0.1 MKCl + 0.25% conc. HCL. Centrifugation (see above) then yields two phases. The lower phase contains the lipids. It is washed with 0.65 vol of CH_2Cl_2/MeOH/H_2O (3/48/47 ; v/v/v) and again centrifuged. The resulting lower phase is collected and concentrated under reduced pressure until near-dry. The residue is dissolved in Toluene/Ethanol (4/1) and stored at -20 °C under Nitrogen or Argon.

Isolation of phospholipid classes by HPLC

Total lipid extracts are dissolved in a mixture of CH_2Cl_2/MeOH (2/1 ; v/v) to a concentrations between 0,5 and 1,5 mol/ml. HPLC was performed on a Varian 5000. Detection at 205 nm (Pey Unicam UV-VIS detector LC3 UV) coupled to a Hewlett Packerd data station (HP 2280 A). For elution a solvent polarity gradient was applied, consisting of acetonitril (Fisons A/0627, HPLC grade) and freshly double-distilled water. The mobile component A was pure acetonitril and component B was a mixture of acetonitril/water (80/20 ; v/v). Eluents were degassed before use.

For analytical runs the following conditions were used:

The separation column, (250 x 4.6 mm I.D.), was packed with Lichrosorb Si

60-5 (Merck, Darmstadt, G.F.R.). This column was protected with a guard column (100 x 3 mm), packed with Lichrosorb Si 60-30 (Merck). Aliquots of 10 µl of the total lipid extract were directly injected without further pretreatment. Up to 150 g lipid can be separated in one run. The columns were equilibrated with 13%B. Gradient elution was performed in four steps: 0-3 min 13%B, 3-15 min 13%B to 75%B linear gradient, 15-35 min 75%B, 35-45 min plateau at 75%B, there after reequilibration of the column with 13%B. A flow-rate of 1 ml/min was used. Eluting peaks were identified by TLC, using standard methods [4].

For preparative separations, conditions were slightly modified:

The separation column dimensions (500 x 22 mm I.D.), was packed with RSil silica 10 micron (Alltech. associates inc, Applied science labs.). The column was equilibrated in 13%B. Maximally 15 mg lipid can be applied in 150 µl injection volume. For elution a five step gradient program was used at a flow rate of 10 ml/min: 0-5 min 13%B, 5-10 min linear gradient 13%B to 65%B, 10-40 min 65%B, 40-45 min linear gradient from 65%B to 80%B. Eluting peaks were identified by TLC and quantified by phosphate analysis [7]. Corresponding fractions were combined, concentration under reduced pressure and stored at -20 ^{o}C under Argon as described for the total lipid extracts.

Fatty acid analysis by GLC

Lipid samples containing up to 0.5 mg phospholipid were dried under a stream of nitrogen and dissolved in 10 l methanol. Susequently 10 µl of the transesterification reagent Methyl Prep II was added (0.2 M M trifluoromethylphenyl trimethylammonium hydroxide in methanol, Applied science Labs.). Samples were incubated at room temperature for 30 minutes. This results in a complete transesterification of the fatty acyl chains to methylesters [8]. Subsequently 1 µl of this mixture was applied for GLC analysis without further purification.

GLC was performed with a Pye Unicam 204, using a prepacked OV 101 column (6 ft x 4mm I.D), and detection by flame ionisation, coupled to a Hewlett Packard data station (HP 2280 A) for automated data processing. The injector and detector temperature were kept at 250 ^{o}C. For elution the following temperature profile was applied: 0-1 min stationairy at 190^{o}C, then increase to 340 ^{o}C at 8 ^{o}C/min. The high final temperature causes some loss (bleed) of stationairy phase material from the column, evident as an increase in the baseline in the last part of the chromatograms. This high temperature however is necessary for elution of the ultra-long fatty acyl methyl esters (> C_{26}).

Figure 1 shows the HPLC profile of the total lipid extract from bovine photoreceptor membranes. The four phospholipid classes phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol can be separated in one run to at least 95% purity. In the analytical mode the phosphatidylcholine fraction appears to be resolved in four subpeaks

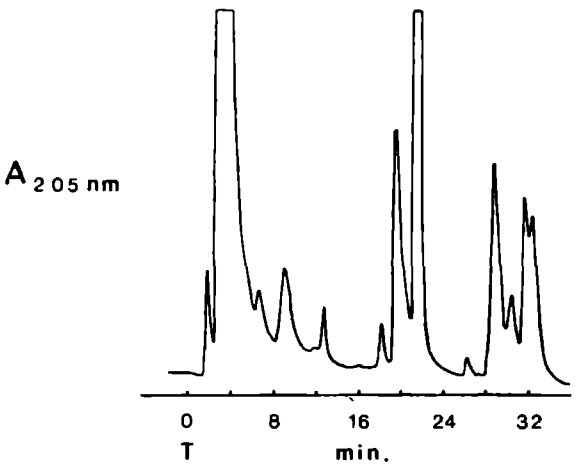
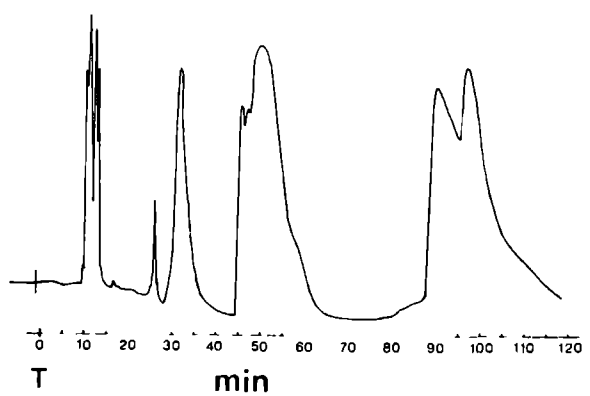


Figure 1 : (A) Typical HPLC elution pattern for bovine photoreceptor membrane total lipid extract in an analytical run. Elution with a linear solvent gradient from 2.5 to 13% (v/v) water in acetonitril, between 3 and 15 min. Flow-rate 1 ml/min. For further experimental details see the materials and methods section. Peaks :
1 = phosphatidylinositol,
2 = phosphatidylserine,
3 = phosphatidylethanolamine,
4 = phosphatidylcholine.



(B) Typical HPLC elution pattern for total lipid extract from bovine photoreceptor membrane in a preparative run. Elution with a two step linear solvent gradient from 2.5 to 13% (v/v) water, between 5 and 10 min and from 13 to 16% (v/v) water, between 40 and 45 min, in an acetonitril-water mixture. Flow-rate 10 ml/min. For further experimental details see the materials and methods section. Peaks :
1 = phosphatidylinositol
2 = phosphatidylserine
3 = phosphatidylethanolamine
4 = phosphatidylcholine

(Fig. 1A) . The preparative mode has somewhat lower resolution and resolves only two phosphatidylcholine peaks (Fig. 1B). The fatty acid composition of the total lipid extract and of the purified phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine fractions was determined by GLC (Fig. 2, Fig. 3, Table 1). The obtained pattern (degree of saturation phosphatidylcholine < phosphatidylethanolamine < phosphatidylserine) and fatty acid composition is in excellent agreement with earlier reports [2,3]. Table 1 furthermore shows that the ultra long fatty acyl chains (> C_{26}) are almost exclusively found in the phosphatidylcholine fraction. The fatty acid composition of the four phosphatidylcholine subpeaks was analysed as well, (Table 2). This nicely demonstrates that elution time increases with increasing saturation. Further it is evident that the first, subfration, which is highly unsaturated, also contains the bulk of the ultra long fatty acyl chains. This suggests that the ultra long chains are poly-unsaturated as well. This could not be confirmed by GLC, since our procedure only discriminates between chain length, but does not resolve species with different levels of unsaturation within one population. However, Aveldano has reported independent evidence that the ultra long chains are indeed mainly of the n: 6 ω 3 type [5].

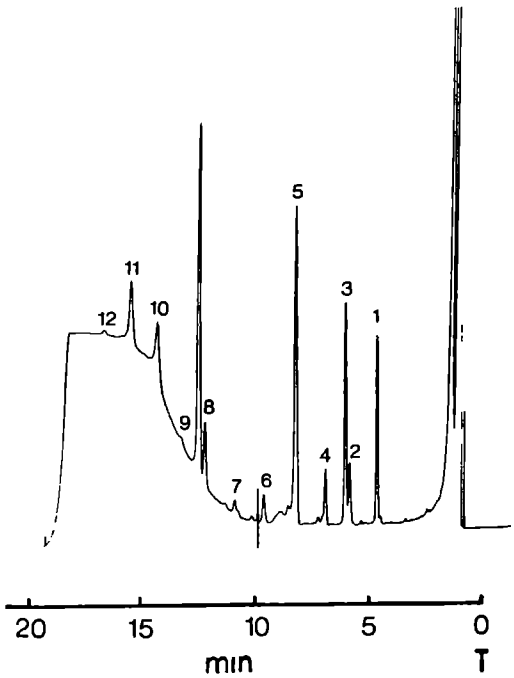


Figure 2 : GLC elution pattern for bovine photoreceptor membrane total lipid extract. Experimental conditions as described in the method section. At * in the chromatogram the detector attenuation was decreased by a factor five for the detection of the very long chain fatty acid methyl esters. Peaks:
1 = 16:0, 2 = 18:1/2, 3 = 18:0,
4 = 20:4, 5 = 22:5/6,
6 = 24:4//5/6, 7 = 26:4/5/6,
8 = 28:4/5/6, 9 = 30:4/5/6,
10 = 32:4/5/6, 11 = 34:4/5/6,
12 = 36:4/5/6.

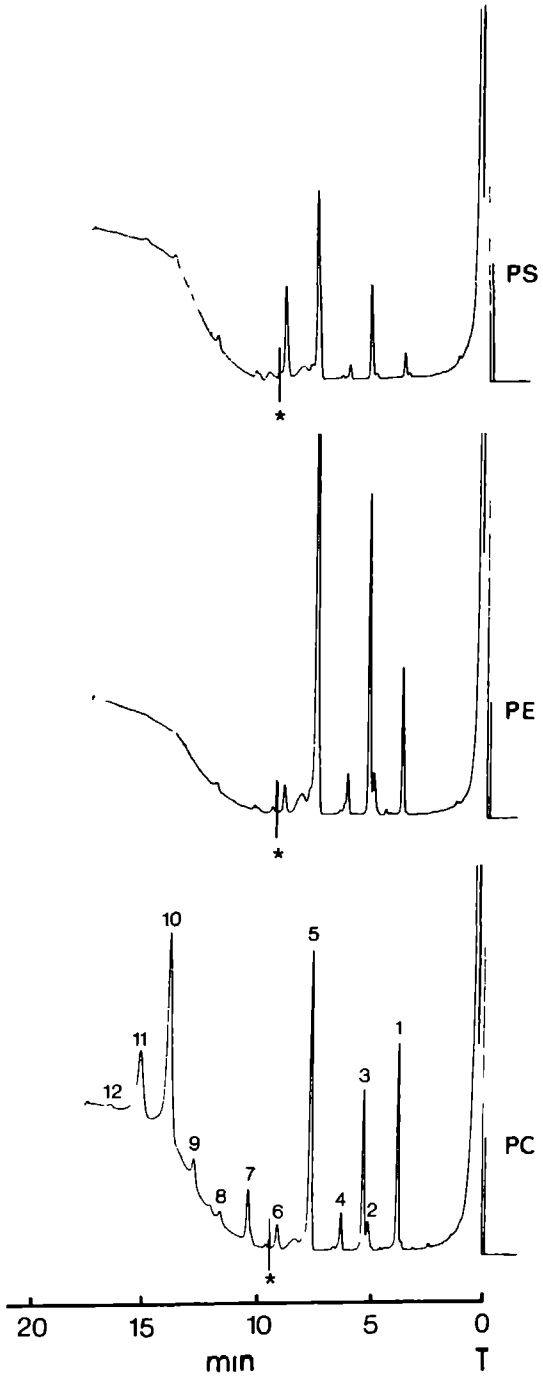


Figure 3 : GLC elution pattern for the photoreceptor membrane phospholipid classes:
(A) phosphatidylcholine, (B) phosphatidylethanolamine, and (C) phosphatidylserine.
Experimental conditions as described in the method section. At * in the chromatogram the detector attenuation was decreased by a factor five for the detection of the very long chain fatty acid methyl esters. Peaks:
1 = 16:0, 2 = 18:1/2, 3 = 18:0,
4 = 20:4, 5 = 22:5/6,
6 = 24:4//5/6, 7 = 26:4/5/6,
8 = 28:4/5/6, 9 = 30:4/5/6,
10 = 32:4/5/6, 11 = 34:4/5/6,
12 = 36:4/5/6.

Table 1: Fatty acid composition of rod outer segments and their major phospholipids. Values are in mol%. PtdSer = phosphatidylserine, PtdEth = phosphatidylethanolamine, PtdCho = phosphatidylcholine. For experimental details see the method section.

Mol% of total phospholipid	(19±2)	(43±1)	(38±2)
Fatty acids	Rod outer segments	PtdSer	PtdEth	PtdCho
16:0	18.3	6.2	12.7	26.0
18:0	21.7	21.9	25.8	18.5
18:1/2	7.1	1.8	4.4	4.7
20:4	6.3	3.2	4.0	4.5
22:5/6	37.5	46.5	50.0	33.3
24:4/5/6	3.4	19.3	3.1	2.4
26:4/5/6	0.4	-	-	1.1
28:4/5/6	1.3	0.6	-	0.2
30:4/5/6	0.2	-	-	0.7
32:4/5/6	1.7	0.3	0.1	5.5
34:4/5/6	1.8	0.3	-	2.7
36:4/5/6	0.2	-	-	0.4

DISCUSSION

The described preparative HPLC procedure allows single run separation of photoreceptor membrane lipids into three phospholipid classes. The latter are obtained in at least 95% purity. Phosphatidylserine and phosphatidylethanolamine elute in a single peak. Phosphatidylcholine is fractionated into at least four subfractions by the analitical column. This is probably due to a difference in polarity resulting from a difference in fatty acyl chain composition (Table 2). Compared to column chromatography or TLC [2,3], our method has several advantages. It is rapid (ca. 100 min. for a complete run) and is easily scaled up (up to 15 mg per run). Furthermore it requires little sample manipulation, which greatly reduces the risk of oxidative damage.

The fatty acid composition (C_{16} - C_{24}) of the three phospholipid classes (Table 1) and of the phosphatidylcholine subfractions (Table 2) agree

Table 2: Fatty acid composition of the subfractions of rod outer segment phosphatidylcholine obtained through HPLC chromatography. Values are in mol%. For experimental details see the method section. PtdCho = phosphatidylcholine.

Mol% of total PtdCho	(37±2)	(13±1)	(21±1)	(28±2)
Fatty acids	PtdCho-A	PtdCho-B	PtdCho-C	PtdCho-D
16:0	14.2	26.5	12.1	33.3
18:0	11.2	21.6	46.7	25.8
18:1/2	7.1	13.4	8.2	9.8
20:4	2.4	4.6	4.3	8.5
22:5/6	45.0	26.3	22.4	21.1
24:4/5/6	2.5	4.3	1.6	1.4
26:4/5/6	1.8	-	0.9	-
28:4/5/6	-	3.3	-	-
30:4/5/6	-	-	2.0	-
32:4/5/6	13.0	-	-	-
34:4/5/6	2.9	-	1.8	-
36:4/5/6	-	-	-	-

closely with those obtained previously by TLC or conventional column chromatography [2,3]. Analysis of these data leads to the conclusion that at least 18% of the photoreceptor membrane phosphatidylcholine must be di-saturated and minimally 25% di-usaturated.

The ultra long fatty acyl chains (C_{26} - C_{24}) are predominantly present in phosphatidylcholine. They are concentrated in subfraction PtdCho-A, which contains 18% acyl chains with length > C_{24}. This fraction contains a high percentage (at least 35 mole%) of dipolyunsaturated phosphatidylcholines. Apparently there is a relative great fraction of phosphatidylcholine molecules that contain two polyunsaturated fatty acyl chains, one of which is of the ultra long type. These unusual phospholipid chains have so far only been detected in photoreceptor membranes. Their structural and functional role is not yet understood. The results of lipid extraction of photoreceptor membranes under several conditions led Aveldano to suggest that these phospholipids may

be strongly associated to rhodopsin [5]. This author proposed that these long chain phospholipids could more easily interact with α-helical sections of rhodopsin. However, the physical properties of phospholipids with such long acyl chains need to be studied.

The primary goal of this work was the development of a simple and reliable method for the isolation of phospholipid classes from photoreceptor membranes, on a preparative scale. The availability of such a method is a prerequisite for further characterization of these phospholipids employing techniques like NMR, fluorescence polarization, and reconstitution studies, which require relatively large amount of material.

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CHAPTER 3

PHASE BEHAVIOR OF ISOLATED PHOTORECEPTOR MEMBRANE LIPIDS IS MODULATED BY BIVALENT CATIONS

Leo C.P.J. Mollevanger and Willem J. De Grip

FEBS Letters (1985) 169, 256-260

INTRODUCTION

Several years ago we demonstrated by means of ^{31}P-NMR and electron microscopy that the photoreceptor membrane shows normal bilayer features under various conditions, but the isolated membrane lipids, resuspended under identical conditions, adopt the hexagonal H_{II} phase together with a lamellar phase interspersed with lipidic particles ("inverted micelles") [1]. Subsequently, authors in [2] confirmed our results with regard to the native membrane, but claimed that the isolated lipids also prefer the lamellar phase. They suggested that this discrepancy with our results might be due to lipid oxidation or incomplete extraction of bilayer-stabilizing species like PtdCho or PtdSer. Analysis of their experimental conditions, however, reveals one major difference with ours: all spectra were obtained in media containing no bivalent cations, while the buffers we used contained physiological concentrations of Ca^{2+} and Mg^{2+} (2 and 3mM, respectively). We have now further investigated this matter and hereby present results which resolve this apparent discrepancy.

MATERIALS AND METHODS

Bovine rod outer segments (ROS) are isolated by sucrose density centrifugation as in [3]. Following hypotonic lysis (0.15 mM Ca-EDTA solution, pH 7.0; 2x) they yield an A_{280}/A_{500} ratio of 2.0-2.2. To obtain lipid extracts free of retinal or retinoxime [1] ROS are isolated in the light [3], which yields a membrane preparation virtually free of retinal and retinol. The SDS-gel pattern, phospholipid and fatty acid composition are checked routinely and found to agree with literature data [4,5]. "Light" or "dark" preparations do not show significant differences in these respects.

Total lipid extracts are obtained by a modified Folch procedure [4]. All manipulations are performed under strict nitrogen-protection with N_2-saturated solutions to minimize oxidative degradation of the highly unsaturated photoreceptor membrane lipids. The final extracts are evaporated in a small round-bottom flask by a stream of nitrogen and subsequently dried at high vacuum on a rotary evaporator for at least 1 h at room temperature to remove the last traces of organic solvent. The lipid residue is rehydrated by adding the required volume of aqueous medium and some glass beads under nitrogen and rotating for 15-30 min (final lipid concentration: 40 umol/ml).

Lipid phosphate (modified Fisko-Subarov [6]) and fatty acid (GLC of methyl esters) are assayed as in [4]. The phospholipid composition is determined both by two-dimensional TLC of the lipid extract [4] and by ^{31}P-NMR in detergent solution [7]. The latter method allows direct assay of the entire membrane and therefore does not depend on lipid isolation.

^{31}P-NMR spectra were recorded at 81 MHz on a Bruker WM-200 spectrometer using broadband bilevel high-power proton decoupling, 0.3 W between pulses (0.8s), 3 W during aquisition (0.25s). Routinely 2000-3000 FIDs were accumulated using 60° pulses, an interpulse time of 0.8s and a 16 kHz sweep width. Before fourier-transformation an exponential multiplication was applied to the accumulated FIDs, resulting in a 50 Hz line broadering. Sample volume was 1.4 ml.

The following two buffer solutions are used. (A) Bivalent cation-free buffer: 20 mM Mops, 0.1 mM EDTA (pH 7.2); (B) Mg/Ca buffer: 20 mM Mops, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2mM $CaCl_2$, 0.1 mM EDTA (pH 7.2). Both buffers are dissolved in H_2O/D_2O (85:15, v/v).

RESULTS

Figure 1 presents a typical ^{31}P-NMR spectrum of washed ROS membranes, showing a characteristic bilayer spectrum [1,2,8].Small percentages of non-bilayer phases (< 1%) may however go undetected in the broad bilayer band. Upon solubilizing the membrane in 5% sodium cholate (pH 8.0), isotropic signals are generated which allows direct calculation of the composition of the major membrane phospholipids (Fig. 2) [7]. The results compare well with analysis of lipid extracts by TLC (table 1). Table 1 further shows that, with regard to phospholipid and fatty acid composition, our preparations are very similar to those analyzed in [2].

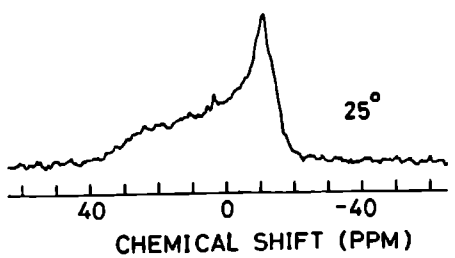

Figure 1 : ^{31}P-NMR spectrum of washed ROS membrane suspended in Ca^{2+} + Mg^{2+} containing buffer B. In the temperature range studied (5-45°C) the spectra show little temperature dependence. Ca^{2+} (tested up to 10 mM) has no influence on the spectrum.

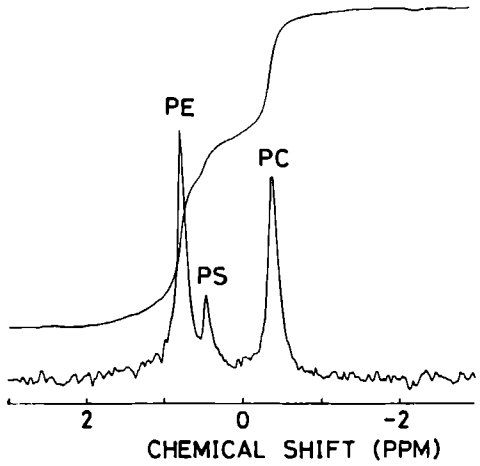

Figure 2 : ^{31}P-NMR spectrum of total rod outer segment lipids redissolved in 5% cholate, 70 mM EDTA in H_2O/D_2O 5:1 (v/v), pH=8, (ca 20 mg lipid/ml). Spectra like this were recorded with gated decoupling and an interpulse time of 5 sec. Integrated spectra (upper curve) are used to calculate the corresponding data in Table 1.

Table 1 : Phospholipid[a] and major fatty acid composition of washed photoreceptor membranes and lipid extracts.

Reference	Lipid origin	Molar percentage (±SD) of			Molar percentage of major fatty acid classes		
		PtdEth	PtdCho	PtdSer	16:0 18:0	20:4, 22:4 22:5, 22:6	24:4 24:5
Here	membrane[b]	43±1	38±2	19±2			
	lipid extract[b]	47±3	40±3	14±2	41	52	1.5
[4]	lipid extract[b]	46.3±1.7	37.8±1.6	15.9±1.0	42	53	1.2
[2]	lipid extract[b]	45.7±0.2	39.8±0.3	14.5±0.2	35	56	3.0

[a] The sum of PtdEth, PtdCho and PtdSer is set at 100%. Minor amounts of other phospholipids (3-5%) are not taken into account.

[b] Determined by ^{31}P-NMR of solutions in 5% cholate. Average of 3 experiments.

[c] Determined by two-dimensional thin-layer chromatography.

Total lipid extracts suspended in the Mg^{2+} + Ca^{2+} containing medium (B) yield spectra as shown in figure 3, which present similar features to those in [1]: an isotropic component, representing a lamellar phase with lipidic particles, superimposed on an anisotropic component with properties of a bilayer below 15°C and of a hexagonal H_{II} phase at temperatures over 20°C. Apparently, these components exchange not at all or only very slowly on the NMR time scale (microseconds). However, suspension of total lipid extract in the buffer solution without free bivalent cations (A) results in spectra as shown in figure 4A, which are very similar to those in [2]: predominantly lamellar phase (> 85%), with only small amounts of isotropic phase. The latter may be due to smaller vesicles or a separate lamellar phase with lipidic particles. These features are relatively independent of the temperature between 5 and 55°C, except that the isotropic peak becomes sharper at higher temperatures (Fig. 4A).

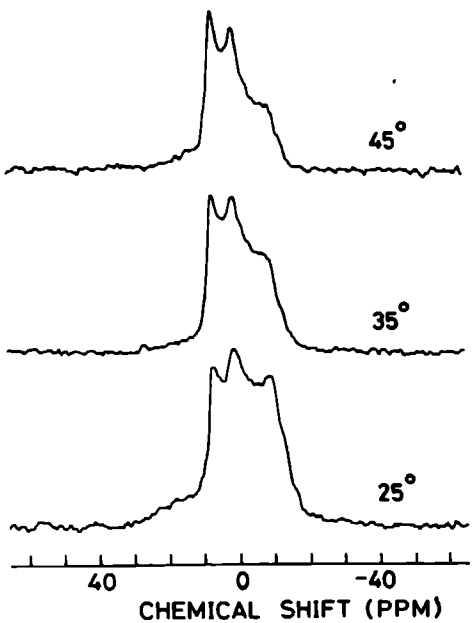

Figure 3 : ^{31}P-NMR spectrum of total ROS lipids, resuspended in Ca^{2+} + Mg^{2+} containing buffer B, as a function of temperature.

This reconciles our earlier results [1] with those in [2]. The following experiment shows that the presence of cations like Ca^{2+} or Mg^{2+} is decisive (Fig. 4B). Upon addition of Ca^{2+} to a suspension of photoreceptor

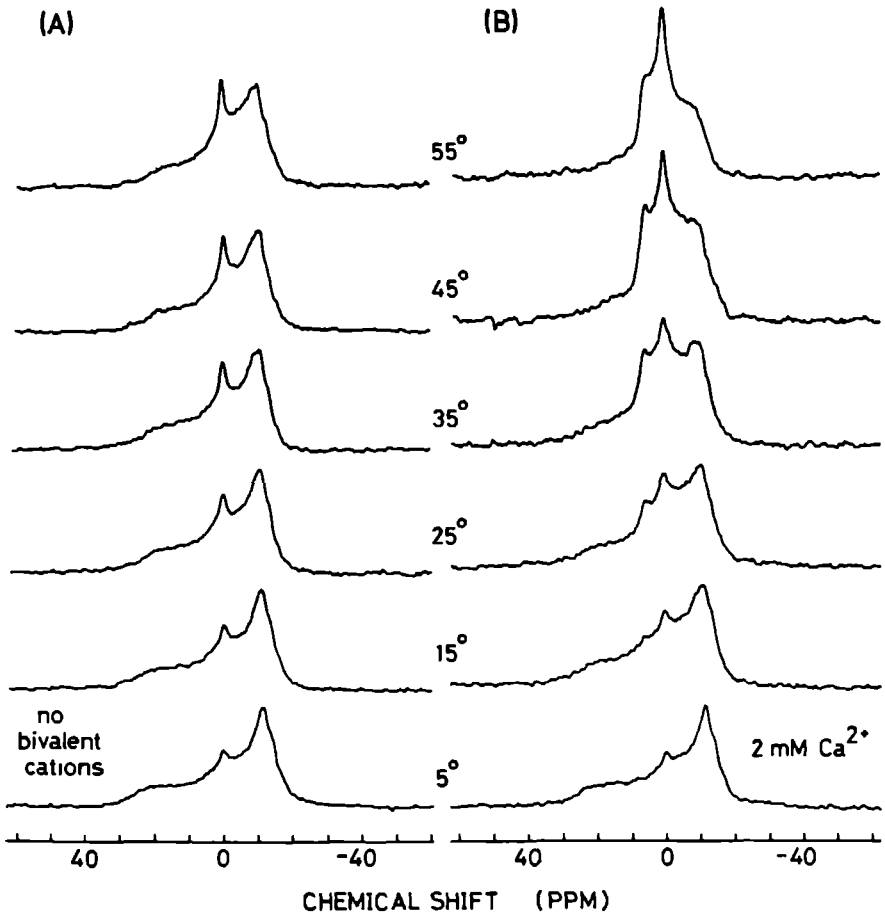

Figure 4 : (A) ^{31}P-NMR spectra of total ROS lipids, resuspended in Ca^{2+}-, Mg^{2+}- free buffer A, as a function of temperature. The sharpening of the isotropic peak is due to a decrease in rotational correlationtime ( ) at in-creasing temperature. The temperature effects are rapidly and completely reversible without any indication of hysteresis. (B) Similar spectra of total ROS lipids resuspended in buffer A, obtained upon addition of $CaCl_2$ at 5°C to a final concentration of 2 mM. This corresponds here to a molar ratio of Ca^{2+} : PtdSer of about 1:3. Starting at 5°C, spectra were obtained after 1 h accumulation, whereupon the sample was warmed up to the next higher temperature, equilibrated for 10 min, and the next accumulation started. The observed effects are not or only slowly reversible by addition of excess EGTA.

membrane lipids in medium A at 25°C no significant effects are observed until the total Ca^{2+} concentration reaches the order of millimolar. This corresponds to Ca^{2+} : PS molar ratios of 0.1-0.2. Then the suspension becomes more turbid and eventually large flaky structures appear. The ^{31}P-NMR spectra show a slow transition to the status observed for total lipid extract suspended directly in solution B. The rate of this transition can be considerably increased by increasing Ca^{2+} concentration or by raising the temperature (Fig. 4B). At 25°C the effects become maximal at 5-6mM Ca^{2+} in this particular case, corresponding to a molar ratio of Ca^{2+} : PtdSer of about 1. Very similar effects are obtained with Mg^{2+} although higher concentrations are required than for Ca^{2+}. Na^{2+} and K^{+} have no effect. Subsequent incubation with excess EGTA only very partially reverses the calcium effect. Even after prolonged incubation (2 weeks) in the presence of EGTA at 4°C to stabilize the lamellar configuration, the isotropic phase remains predominant.

The various manipulations do not significantly influence the fatty acid composition of the lipids, indicating absence of oxidative degradation.

DISCUSSION

Evidently, the extracted photoreceptor membrane lipids do largely adopt the lamellar configuration when resuspended at physiological pH in aqueous solution containing no bivalent cations. This confirms the results in [2]. However, addition of bivalent cations like Ca^{2+} or Mg^{2+} in millimolar concentration induces dramatic phase changes which proceed rapidly at physiological temperatures. This confirms our earlier results [1] and reconciles those in [1,2].

The question remains how this phase change is induced on a molecular level. It is well documented that in aqueous media at neutral pH unsaturated PE prefers the hexagonal phase at physiological temperatures, while under these conditions both PtdCho and PtdSer only adopt the lamellar phase [8]. To generate PtdEth-containing bilayer membranes at temperatures above its transition to the H_{II} phase, at least an equal amount of a "bilayer-stabilizing" phospholipid like PtdCho or PtdSer has to be incorporated [8,9]. Furthermore, there is ample evidence that PtdSer has a high affinity for cations (bivalent >> monovalent; cf. [10]). In bilayers of pure PtdSer, cations like Mn^{2+}, Ca^{2+} and Mg^{2+} induce a strong rigidification [11],

while in mixtures of PtdSer with other lipids (PtdEth or PtsCho) such cations induce a lateral segregation (cf. [8,12,13]), leading to two separate lipid domains containing largely PtdSer, and largely PtdEth or PtdCho. In the case of PtdSer/PtdEth mixtures, the PtdEth domain then may adopt the hexagonal phase [8]. Mixtures of the more saturated major erythrocyte inner monolayer phospholipids (PtdEth,PtdCho,SM,PtdSer) with equimolar amounts of cholesterol also show a phase change from bilayer to hexagonal upon addition of Ca^{2+} [14].

Although alternative explanations may be devised, we consider, in view of the findings summarized above, the following one the most likely for the observed cation effects on the phase behavior of the highly unsaturated photoreceptor membrane lipids. In the absence of bivalent cations the lipids largely adopt the bilayer configuration, since the amount of PtdCho and PtdSer together (40% + 14%) is sufficient to stabilize a bilayer with 47% PtdEth present. However ,addition of Ca^{2+} or Mg^{2+} induces a lateral segregation creating separate PtdSer domains, thereby withdrawing PtdSer from the delicate equilibrium between PtdCho + PtdSer on the one hand and PtdEth on the other. In the rest of the bilayer, largely containing PtdCho and PtdEth now, the ratio PtdCho : PtdEth is too low to stabilize the bilayer configuration. As a result, part of the PtdEth will enter the hexagonal H_{II} phase. The remainder of the PtdEth and PtdCho might create a lamellar phase with lipidic particles and intermembrane contacts [1,8], permitting rapid equilibrium of the lipids over the two bilayer leaflets, which will generate an isotropic ^{31}P-NMR signal. This process is accelerated by raising the temperature, since (1) at temperatures below 15°C the lamellar organization is the most stable one also for PtdEth, (2) lipid mobility and consequently the rate of phase separation and phase transition increases at higher temperatures. The rate of the phase change increases with increasing Ca^{2+} concentration up to a molar ratio of Ca^{2+} : PtdSer of about 1, which indicates that formation of a Ca-PtdSer complex is an essential element in the process and may be rate-determining. The fact that the transition is not easily reversible has also been observed in other systems, such as PtdSer/PtdEth mixtures [8]. Presently, we are testing the above hypothesis by purification of the phospholipid classes to recombine them in different ratios and to investigate cation effects on such systems.

Our observations have several implications for photoreceptor membrane structure and function. The Ca^{2+} content of the disk is relatively high

[15], while intracellular (Mg^{2+}) very likely is in the millimolar range. Hence, irrespective of the transmembrane distribution of phospholipid species, under such conditions the extracted lipids would not prefer the lamellar configuration observed for the intact membrane. This implies that a bilayer-stabilizing factor has to be present in the membrane, for which the major (> 90%) membrane protein rhodopsin is a likely candidate . As an intriguing consequence, protein-configuration changes or local changes in cation concentration during visual excitation might allow functional, and transient local changes in lipid organization (e.g., the lipidic particles may considerably increase ion permeability). Such changes, which may effect only a small part of the total lipid population and hence may easily escape detection, clearly require further study.

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CHAPTER 4

POLYMORPHISM OF PHOTORECEPTOR MEMBRANE LIPIDS : CONTRIBUTIONS OF PHOSPHATIDYLETHANOLAMINE, PHOSPHATIDYLSERINE AND CHOLESTEROL.

L.C.P.J. Mollevanger and W.J. De Grip.

Submitted for publication

The primary action in the visual process takes place in the stack of photoreceptor membranes, located in the outer segment of the rod photoreceptor cell. This photoreceptor membrane has two characteristic features 1) It contains one predominant membrane protein in high density ($2*10^4/um^2$), i.e. the photoreceptor protein rhodopsin. 2) Its lipid components show an unusual high degree of unsaturation. Only 40% of the lipid fatty acyl chains are saturated, while more than 55% consists of long chain fatty acids with at least four, but mainly six unsaturated bonds [1,2,3]. These polyunsaturated fatty acyl chains predominate in the phospholipids phosphatidylserine and phosphatidylethanolamine, which comprise about 16% and about 40% of the total lipid content respectively. Of this population even more than 25% is dipolyunsaturated [1].

It is well documented that the degree of unsaturation modulates the polymorphic phase behaviour of certain lipids, i.e. their tendency to generate different 3-dimensional aggregation modes in aqueous environment [4,5,6]. Thus, unsaturated phosphatidylethanolamine is capable of packing into non-bilayer structures like the hexagonal H_{II} phase. The transition temperature between the lamellar phase and the hexagonal H_{II} phase decreases with increasing unsaturation [4]. Furthermore, interaction of negatively charged phospholipids (phosphatidylserine, phosphatidylglycerol, phosphatidic-acid and cardiolipin) with divalent cations or protons can also induce polymorphic phase behaviour in these lipids. These effects are again enhanced for increasing degrees of unsaturation [6]. Finally, neutral lipids like diglycerides and cholesterol may also stimulate the formation of non-bilayer structures [7,8]. On the basis of such evidence, one would expect the lipid matrix of the photoreceptor membrane to have a strong tendency to polymorphic phase-behaviour in view of their high content of polyunsaturated phosphatidylethanolamine, and appreciable amounts of polyunsaturated phosphatidylserine and cholesterol [1,2]. Indeed, previous work demonstrated that polymorphism is induced at physiological temperature in the total population of isolated photoreceptor membrane lipids (i.e. in the absence of rhodopsin) by the presence of the divalent cations Ca^{2+} and Mg^{2+} [9,10,11]. Which components in the lipid matrix primarily contribute to this behaviour has so far not been elucidated, although the interaction of phosphatidylserine with divalent cations has been proposed to act as a trigger

[10].

Non bilayer phases, in particular the "inverted micellar" intermediate between the lamellar and hexagonal superstructure, have been put forward to participate in very important physiological processes like membrane fusion (endocytosis, exocytosis, membrane biogenesis, cell division), protein insertion or translocation through a membrane (functional in protein biosynthesis) and lipid translocation [12,13]. As a matter of fact these processes are very important in the rod cell, where rhodopsin is biosynthesized at a constant high rate and where biogenesis of photoreceptor membranes takes place continuously at very specific target sites [14,15].

In order to determine which factors primarily contribute to the polymorphic phase behaviour of the photoreceptor membrane lipids, we have investigated the phase behaviour of the total lipid population and the individual phospolipid classes in dependence of increasing Ca^{2+} concentration. The polymorphism of the total lipid matrix appears to originate in the strong tendency of the phosphatidylethanolamine component to organize in the H_{II} phase but is only effectuated upon formation of 1:1 calcium-phosphatidylserine complexes, which per se are very fusogenic. The presence of cholesterol indeed lowers the bilayer to non-bilayer transition temperature.

MATERIALS AND METHODS

Isolation of rod outer segment membranes

Bovine eyes were obtained from the local slaughterhouse. Within two hours post mortem the retinas were excised, homogenized and the rod outer segments purified by sucrose density gradient centrifugation essentially as described by De Grip et al. [16]. If dark adapted membranes were required all manipulations were carried out under dim red light ($\lambda >$ 650 nm). For light adapted membranes the procedure was carried out under day light and NADPH was added to stimulate enzymatic conversion of liberated retinal into retinol [16]. Following two washings in hypotonic buffer (5 mM EDTA, pH = 7.2) the rod outer segment membranes yield typical A_{280}/A_{500} ratios of 2.0-2.2 upon spectroscopic analysis [16].

Lipid preparations

Total lipid extracts were obtained from washed rod outer segment membranes by a modified Folch extraction [2]. Dark adapted membranes were

mixed, before extraction, with an equal volume of 0.1 M hydroxylamine pH = 7.2, and exposed to light (20 min, 300 W tungsten lamp equipped with a 530 nm Schott-Jena cut-off filter) in order to convert retinal into retinal oxime [17]. Light adapted membranes were extracted directly. Both procedures yield similar results. The phospholipids were purified from the neutral lipids by conventional column chromatography of the total lipid extract over silicic acid using chloroform as eluent [17]. The phospholipid classes phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine were purified by straight-phase HPLC of total lipid extracts (Si 60-5 (Merck , Darmstadt, GFR); analytical scale 250 mm x 4.6 mm, preparative scale 500 mm x 22 mm). For elution, a linear gradient of acetonitril-water mixtures, 100:26 to 100:15, was used as described by Nissen and Kreysel [18]. Eluting lipids were monitored at 205 nm (Pye Unicam UV-VIS detector LC3-UV). On a preparative scale this procedure affords a good separation of phospholipid classes up to a sample size of 15 mg [3]; see chapter 2.

All manipulations with the lipids were performed under a nitrogen atmosphere to protect the poly-unsaturated acyl chains from oxidative degradation. Lipid preparations were stored at -80 ^{o}C under Argon. Regular checks on the fatty acid composition of the purified native membrane and lipid preparations were performed by GLC as described before [2]. Phospholipid contents were determined by phophate analysis using a modified Fiske-Subarov procedure [19]. This phospholipid composition was routinely checked by two dimensional TLC according to Drenthe et al. [2].

NMR measurements

^{31}P-NMR measurements were carried out on a Bruker WM 200 NMR spectrometer operating at 81 MHz and interfaced to an Aspect 2000 computer. Observation pulses of 15 s duration, corresponding with a 60 degree pulse angle were used in the experiments. During the measurements bi-level proton decoupling was employed i.e. 3 W during the data acquisition period (0.12 s) and 0.3 W during relaxation delay. The time between observation pulses amounted to 1 s. Typically 2000-3000 transients were accumulated for each spectrum. Temperature was controlled by a stream of thermostatted air. Measurements at different temperatures were performed under computer control. After each temperature change the sample was equilibrated for 10 min before acquisition started. To improve the signal to noise ratio in the spectra an exponential multiplication corresponding to a 50 Hz line broadening was applied to the accumulated free induction decays, before Fourier

transformation.

All NMR samples were suspended in : 20 mM Mops, 0.1 mM EDTA, dissolved in H_2O/D_2O (85:15, v/v) pH = 7.2. Total sample volume was typically 1.5 ml (10 mm NMR tube). Lipids were resuspended, after thorough removal of organic solvent (1 hour under high vacuum), by mechanical agitation under an Argon atmosphere. All samples typically contained 30-50 mg of lipid material/ml. Stepwise increase of Ca^{2+} concentration in 1 mM steps was realized by subsequent addition of aliquots of a concentrated Ca^{2+} solution (60 mM) to the lipid suspension in the NMR tube. Following every addition at least two successive NMR spectra were recorded to ascertain that any changes following Ca^{2+} addition had stabilized.

RESULTS

Total rod outer segment membrane lipids: effect of Ca^{2+} concentration

It has previously been shown by ^{31}P-NMR spectroscopy and electron microscopy that millimolar concentrations of Ca^{2+} and Mg^{2+} induce formation of inverted micelles and hexagonal H_{II} phase in isolated rod outer segment membrane lipids at temperatures above 25 °C [9,10,11]. We have further investigated this cation effect by means of ^{31}P-NMR. The impact of a stepwise increase of the Ca^{2+} concentration on the phase behaviour of these lipids was analised. As demonstrated before, the bilayer configuration of the lipid matrix in the intact photoreceptor membrane is still not perturbed by Ca^{2+} ions at temperatures as high as 55 °C (Fig. 1) [10,11]. The isolated lipids react strikingly different. In the absence of multivalent cations, ^{31}P-NMR spectra of these lipids show the typical bilayer lineshape up to at least 55 °C [10]. In the presence of 2 mM Ca^{2+} however signals with non-bilayer lineshapes appear between 20 and 25 °C and increase in intensity at increasing temperatures [10,11]. When the temperature is kept constant at 25 °C, increasing Ca^{2+} concentrations gradually convert the lineshape characteristic for a pure bilayer, in the absence of Ca^{2+} ions, to a mixture of lineshapes, reflecting the presence of 85-90% hexagonal H_{II} phase at Ca^{2+} concentrations over 12 mM (Fig. 2). This is also evident in electron micrographs which show the presence of typical oriented cylinders of the hexagonal H_{II} phase [9,11]. The bilayer to hexagonal transition cannot be reversed by addition of an excess of the strong Ca^{2+} complexing agent EGTA,

and/or incubation at a temperature (4°) C below the bilayer-hexagonal H_{II} transition temperature (20°).

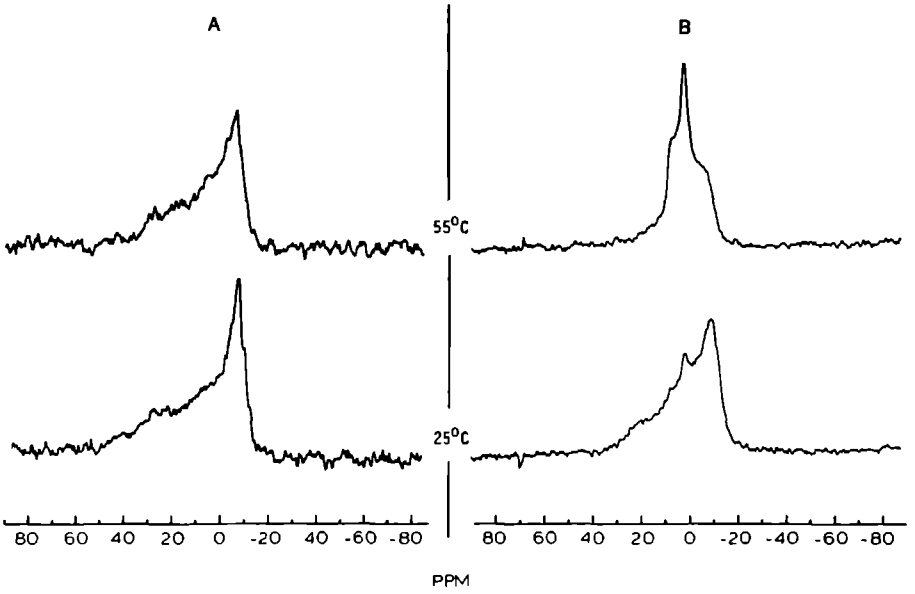

Figure 1 : Broadband ^{1}H decoupled ^{31}P-NMR spectra of photoreceptor membranes (A) and their total lipid extract (B) at 25 and 55 °C in the presence of Ca^{2+}. Solution conditions: (A) 16 mM Ca^{2+}, 20 mM Mops, pH=7.2 ; (B) 2 mM Ca^{2+}, 20 mM Mops, pH=7.2. In both experiments the sample contained about 50 mM phospholipid.

A plot of the relative percentage of hexagonal H_{II} phase versus the Ca^{2+} concentration shows a linear relationship in the first part of the titration curve (Fig. 3). The curve levels of at Ca^{2+} concentrations about equivalent to the molar concentration of phosphatidylserine. A similar relationship has been observed repeatedly at various phospholipid concentrations. This suggests that for maximal conversion of a bilayer into a hexagonal assembly an 1:1 Ca^{2+} to phosphatidylserine ratio is required and that fractional amounts of this ratio only lead to conversion of an equivalent fraction of the bilayer phase. Complete conversion to the hexagonal H_{II} phase is not observed however. Even at a five-fold excess of Ca^{2+} to phosphatidylserine, about 10 to 15% of the intensity in the NMR spectrum retains a bilayer like lineshape.

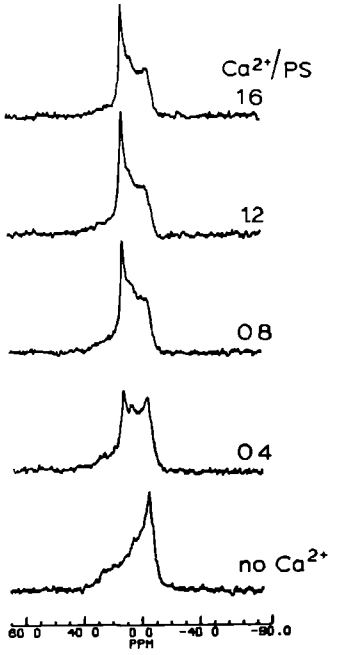

Figure 2 : Broadband ^{1}H decoupled ^{31}P-NMR spectra recorded at 25 °C of a dispersion of total lipid extract from photoreceptor membranes as a function of calcium concentration. The numbers indicate the calcium/phosphatidylserine ratio at which the spectrum was measured Sample conditions: 20 mM Mops buffer, pH=7.2 and 10 mM phosphatidylserine.

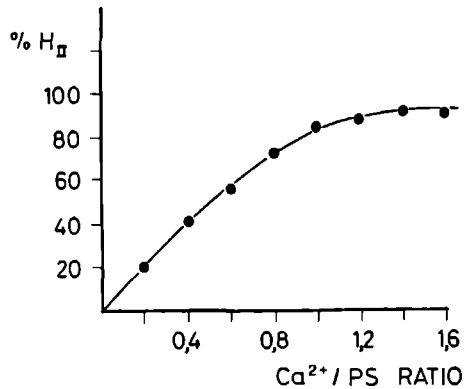

Figure 3 : Plot of the percentage hexagonal H_{II} phase deduced from the ^{31}P-NMR spectra of total lipid extracts of photoreceptor membranes measured at 25 °C as a function of the molar ratio calcium/phosphatidylserine.

Total phospholipids still show polymorphic phase-behaviour

There is ample evidence that cholesterol can promote hexagonal phase formation, in particular in mixed lipid systems containing unsaturated acyl chains [8]. Although photoreceptor membranes have a relatively modest cholesterol content, ca. 12% on a molar basis, a possible impact of this

constituent deserves attention. Therefore the phospholipids in the total lipid extract were separated from the neutral lipids by column chromatography over silicic acid [17], and analysed for their polymorphic phase behaviour by the same approach used for the total lipids (Fig. 4). In the absence of Ca^{2+}, the total phospholipid population again assembled only into bilayers up to the

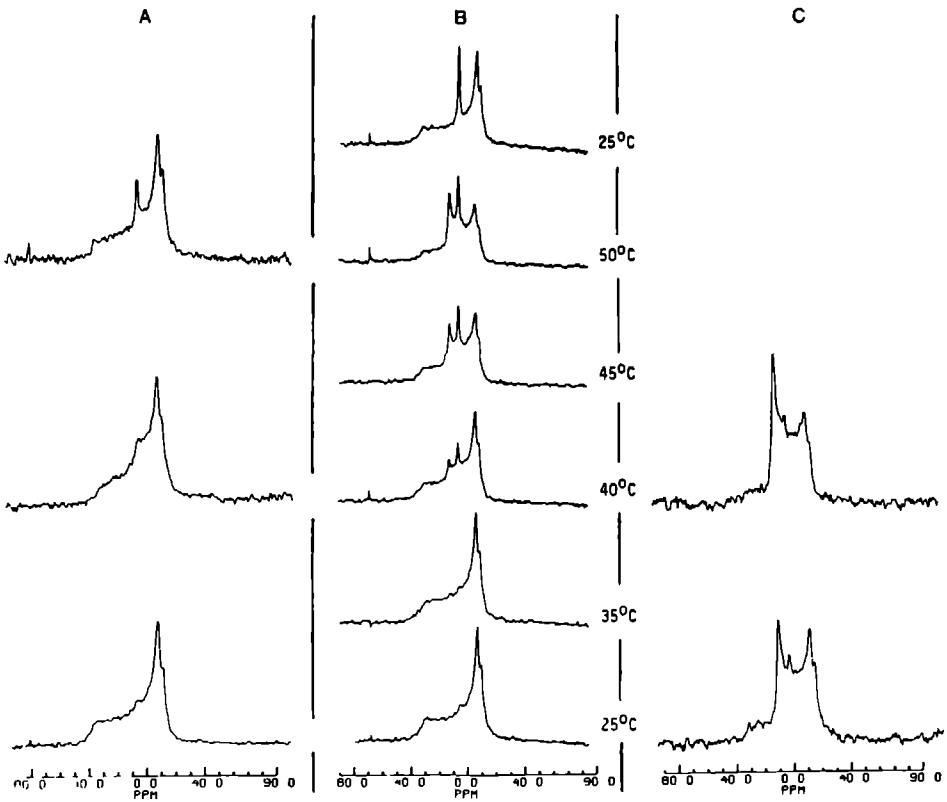

Figure 4 : Broadband ^{1}H decoupled ^{31}P-NMR spectra of the total phospholipid population of photoreceptor membranes in the absence (A) or the presence (B) of excess calcium (molar ratio calcium/phosphatidylserine 1.5:1) as a function of temperature. (C) Similar to (B) but in addition contained 12 mole % cholesterol. Solution conditions: 20 mM Mops buffer, pH=7.2, 50 mM phospholipid. The top spectrum in (B) was obtained after recooling the sample from 50 °C back to 25 °C and shows that the hexagonal component under these conditions reverses to bilayer and isotropic components

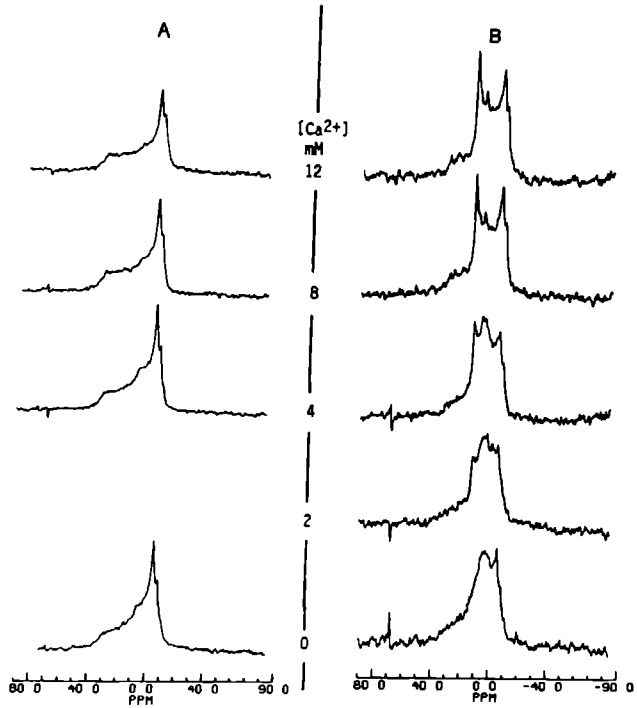

Figure 5 : Broadband ^{1}H decoupled ^{31}P-NMR spectra recorded at 25 ^{o}C, of the total phospholipid polpulation of photoreceptor membranes in the absence (A) and presence (B) of 12 mole % cholesterol, as a function of the calcium concentration. Solution conditions: 20 mM Mops, pH=7.2, 50 mM phospholipid, corresponding to 9 mM phosphatidylserine.

highest temperature measured (55 ^{o}; Fig. 4A). Addition of Ca^{2+} ions induced formation of non bilayer phases, but compared to total lipid population the transition temperature was shifted at least 15 ^{o} upward, to about 40 ^{o}C (Fig. 4B). At 25 ^{o}C, addition of Ca^{2+} could not promote detectable formation of non bilayer phases (Fig. 5A). Only at temperatures over 35 ^{o}C the phospholipid bilayer is no longer stable in the presence of Ca^{2+} (Fig. 4B). Under these conditions again the maximal effect is observed at a molar ratio of Ca^{2+} to phosphatidylserine of at least 1, and addition of EGTA did not result in reversion to the bilayer. These results suggest that the lower thermal stability of the bilayer in the total lipid extract is indeed due to the presence of cholesterol. Therefore cholesterol was added

back to the isolated photoreceptor membrane phospholipids up to the natural molar ratio of 12%, and the phase-behaviour of this mixture was investigated (Fig. 4C, 5B). The mixed cholesterol-phospholipid vesicles gave no indication for non bilayer phases in the absence of Ca^{2+}, but the Ca^{2+}-induced transition of the bilayer to hexagonal H_{II} assembly now again starts at about 20 ^{o} (figure 4, 5). This is comparable to the transition temperature found for the photoreceptor membrane total lipid extract but 15 ^{o}C lower than that for the isolated phospholipids without cholesterol.

Phase behaviour of the individual phospholipid classes

In order to verify the role of the individual phospholipid classes, these were purified by means of preparative HPLC. The obtained fractions were at least 95% pure, according to quantitative two-dimensional TLC. The fatty acid composition of the fractions confirmed earlier reports [1,2]. The order of unsaturation decreases in the order phosphatidylserine > phosphatidyl-ethanolamine > phosphatidylcholine.

According to the literature [12,13,20] phosphatidylcholine always assembles into regular bilayers irrespective of its degree of unsaturation. Liposomes prepared from the purified photoreceptor membrane phosphatidylcholine fraction indeed behaved quite accordingly upon visual inspection and were not further examined by ^{31}P-NMR spectroscopy. On the other hand, the highly unsaturated phosphatidylethanolamine population (57% of the fatty acyl chains are polyunsaturated) is expected to adopt the hexagonal H_{II} phase spontanuously at subzero temperature, in analogy to synthetic poly-unsaturated model systems [20]. This was confirmed by ^{31}P-NMR spectroscopic analysis of dispersions of the phosphatidylethanolamine fraction at 0 ^{o}C (Fig. 6A). It produces the characteristic hexagonal H_{II} lineshape with a low-field shoulder and high-field peak and a chemical shift anisotropy of about 24 ppm. A minor component (< 10%), giving rise to the signal at the isotropic position, represents very small vesicular or micellar structures, which allow rapid isotropic movement of the phospholipid molecules ($\tau < 10^{-3}$ sec). The presence of Ca^{2+} ions (up to 10 mM) had no detectable effect on the lineshape of the NMR spectra obtained for this phospholipid.

The phosphatidylserine fraction, which is extremely unsaturated (70% polyunsaturated fatty acyl chains), exhibited an unusual behaviour. First of all it readily resuspended in aqueous buffer at neutral pH. In the absence of divalent cations, the resulting suspension showed little turbidity and the

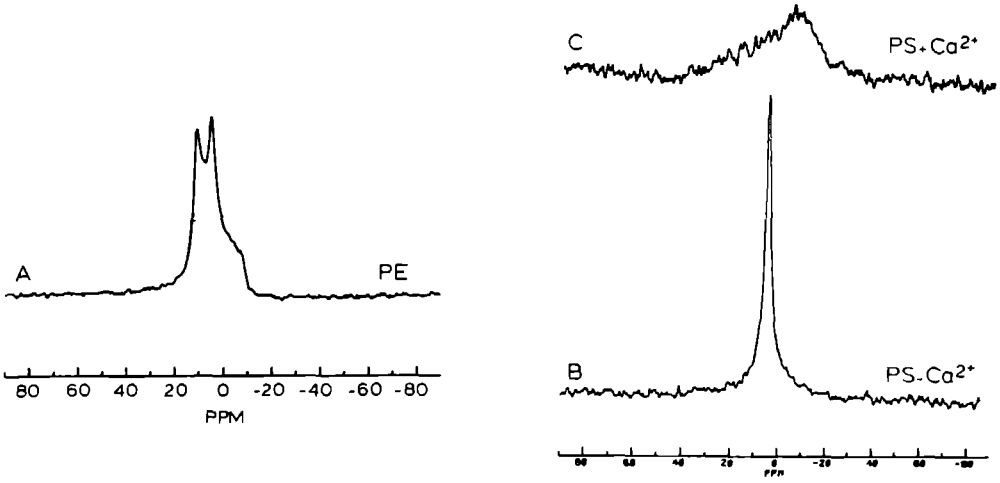

Figure 6 : ^{31}P-NMR spectra (broadband ^{1}H decoupled) of purified phospholipid fractions of the photoreceptor membrane dispersed in 20 mM Mops, pH=7.2. (A) Phosphatidylethanolamine dispersed and recorded at 0 °C. (B) Phosphatidylserine without calcium ions at 25 °C. (C) As in (B) but following addition of calcium ions to a molar ratio calcium/phosphatidylserine of 1.5:1. With respect to spectrum (B) the amplitude of spectrum (C) was multiplied by a factor of 2.

^{31}P-NMR spectrum only revealed a narrow resonance at the isotropic chemical shift position (Fig. 6B). These characteristics are typical for very small vesicular structures ($\phi <$ 100 nm) [21]. Upon addition of Ca^{2+} ions, the narrow isotropic resonance converted into the broad lineshape characteristic for phospholipids in extended fluid bilayers (Fig. 6C). The fusogenic capacity of vesicles containing phosphatidylserine in the presence of Ca^{2+} is well documented [22]. Clearly Ca^{2+} ions also induced fusogenic reactions in vesicles consisting of pure photoreceptor membrane phosphatidylserine, leading to a marked increase in vesicle size. There is substantial evidence that more saturated phosphatidylserine species immobilize in the presence of Ca^{2+} ions into rigid Ca^{2+}-phosphatidylserine complexes known as cochleate cylinders [12,23,24]. In terms of ^{31}P-NMR spectroscopy such an immobilization of the phospholipid molecules means broadening of the signal from approximately 50 ppm (for a liquid crystalline bilayer) to around 200 ppm (for gel like structures), which practically results in complete loss of the ^{31}P-NMR signal. Assuming that the relaxation time is similar for both systems, loss of

^{31}P-NMR signal upon going from figure 6B to 6C is less than 40%. Hence the photoreceptor membrane phosphatidylserine bilayer does not markedly rigidify in the presence of Ca^{2+} ions.

DISCUSSION

Origin of the polymorphism of photoreceptor membrane lipids

On the basis of studies with highly unsaturated model compounds [5,20] it was suggested that phosphatidylethanolamine is probably involved in the polymorphic phase-behaviour of isolated photoreceptor membrane lipids [9,10]. Our present evidence provides irrefutable experimental proof for this suggestion. The observed polymorphism is indeed carried by the phosphatidylethanolamine fraction in the photoreceptor membrane lipids. This fraction already prefers the hexagonal H_{II} organization at 0 °C (Fig. 6A). The transition range for the bilayer to hexagonal H_{II} conversion probably lies at still lower temperatures, since di(18:2)phosphatidylethanolamine is reported to convert below -15 °C [5], and di(20:4)phosphatidylethanolamine below -30 °C [20].

This property of phosphatidylethanolamine is however not sufficient by itself to to produce non-bilayer configurations in aqueous dispersions of total photoreceptor membrane lipids. Both the phosphatidylcholine and phosphatidylserine fraction only generate the lamellar phase at physiological pH, in agreement with model studies of less poly-unsaturated species [12,13,20]. Together these lipids comprise about 55 mol% of the phospholipid population and this 1.4 molar excess over phosphatidylethanolamine is apparently sufficient to increase the activation barrier for bilayer destabilization to such an extent that even at 55 °C only lamellar phase is observed [10,11]. Model studies on lipid mixtures have very elegantly demonstrated that phospholipids such as phosphatidylcholine and phosphatidylserine, which prefer a bilayer organization can indeed prevent phosphatidylethanolamine from adopting the hexagonal H_{II} phase [12,24,25]. In minimally unsaturated lipids, ratios of 0.4 - 0.8 (bilayer stabilizing lipids relative to phosphatidylethanolamine) are sufficient to maintain the bilayer phase. In systems containing more unsaturated acyl chains higher amounts of bilayer stabilizing lipids are required, up to equimolar ratios [24,25]. With regard to the photoreceptor membrane lipids we have not determined the minimal ratio of phosphatidylcholine or phosphatidylserine to

phosphatidylethanolamine required to maintain a bilayer organization of the phosphatidylethanolamine from the photoreceptor membrane, but the Ca^{2+}-effect discussed below suggests that for this poly-unsaturated system a 1 : 1 ratio phosphatidylcholine to phosphatidylethanolamine is still not sufficient.

In order to expose the latent preference of the phosphatidylethanolamine fraction for the hexagonal H_{II} phase, bivalent cations like Ca^{2+} or Mg^{2+} are required in millimolar concentration. Studies on model membranes containing mixtures, consisting of minimally unsaturated (1 double bond per acyl chain) lipids, including phosphatidylethanolamine and phosphatidylserine, have demonstrated that addition of millimolar concentrations of Ca^{2+} may lead to bilayer destabilization due to lateral segregation of immobilized calcium-phosphatidylserine complexes [27]. Usually molar ratios of Ca^{2+} to phosphatidylserine of 0.5 to 1.0 already produce the maximal effect, although in more complex mixtures containing more unsaturated lipids ratios of 1 to 1 and/or the presence of cholesterol may be required [27,28]. One would therefore expect that the Ca^{2+} induced polymorphism in the isolated photoreceptor membrane lipids also relies on the formation of Ca^{2+} phosphatidylserine complexes. However, a study on the interaction of Ca^{2+} with photoreceptor membrane phosphatidylserine, using fluorescence polarization with parinaric-methyl ester as a probe, showed a slight increase in the gel to liquid crystalline transition temperature but no effect on the fluidity of the system at temperatures over 15 ^{o}C [29]. These authors concluded that the Ca^{2+}-phosphatidylserine interaction is much "weaker" than in less unsaturated phosphatidylserine species. While the lineshape of our ^{31}P-NMR spectra (Fig. 6C) indeed confirms that the majority of the phosphatidylserine fraction remains organized in a fluid bilayer following addition of Ca^{2+}, the avid fusogenic behaviour of the phosphatidylserine vesicles in the presence of millimolar Ca^{2+} concentrations is very similar to that of less unsaturated species [30]. Further, an early analysis of the affinity of isolated photoreceptor membranes for Ca^{2+} by equilibrium dialysis produced apparent dissociation constants which were comparable to egg phosphatidylserine (1-2 mM) [31,32]. Recently we investigated the interaction of Ca^{2+} with hypotonically lysed photoreceptor membranes as well as their isolated total lipid fraction, using Ca^{2+} electrodes to measure the bulk free Ca^{2+} concentration [33]. We arrived at similar apparent dissociation constants (1-2 mM) as reported by Hendriks et al. [31], and the total number

of binding sites was equivalent to the amount of phosphatidylserine present (1.0 ± 0.2, n=5). Hence it appears that the phosphatidylserine fraction in the photoreceptor membrane lipid population interacts normally with Ca^{2+} ions, but the voluminous poly-unsaturated fatty acyl chains prevent close enough packing to form stable Ca^{2+}-bridges which causes the remarkable rigidification observed in less unsaturated species [23,28].

This conclusion leaves two major questions: 1) How does formation of these single Ca^{2+}-phosphatidylserine units suppres the bilayer stabilizating action of phosphatidylserine. 2) What factors are responsible for the observation that in the intact photoreceptor membrane, even under extreme conditions (50 mM Ca^{2+}, 55 ^{o}C), no perturbation of the bilayer is detectable [9,10,11]. The second question is addressed in a separate paper [33]. The first question is discussed in the following paragraph.

On the mechanism of Ca^{2+} induced bilayer destabilization in the isolated photoreceptor membrane lipids

In aqueous dispersion of pure phosphatidylethanolamine the transition of bilayer to the hexagonal H_{II} phase is a thermal process with a relatively small transition enthalpy (ca 7 kJ/mole) [20]. The transition temperature decreases with increasing unsaturation. In this system the presence of cholesterol has relatively little effect [27]. In mixed lipid systems containing phosphatidylethanolamine, cholesterol may considerably lower the transition temperature [28]. We observe the same effect in the isolated phospholipid fraction of the photoreceptor membranes, where in the presence of cholesterol the Ca^{2+} induced lamellar to hexagonal H_{II} phase transition appears at a temperature 15 to 20 ^{o}C lower than found for the pure phospholipids. It has indeed been demonstrated that cholesterol affects the thermotropic behaviour of the photoreceptor membrane phosphatidylcholine fraction in the usual way [29,34].

Bilayer destabilization in vesicles of isolated photoreceptor membrane lipids is however not just a thermal process, but requires the assistence of divalent cations. The picture of lateral segregation of calcium-phosphatidylserine complexes and formation of rigid calcium-phosphatidylserine patches, leaving a phosphatidylethanolamine /phosphatidylcholine mixture with insufficient bilayer stabilizing capacity of phosphatidylcholine [27], probably is too simple for this poly-unsaturated system. Tilcock et al. [28] observed no immobilization of phospholipid in

mixed lipid systems of phosphatidylethanolamine/phosphatidylcholine/phosphatidylserine and cholesterol containing phosphatidylserine with di-unsaturated fatty acyl chains upon addition of calcium. This in contrast to the mono-unsaturated species. Our results indicate that even the isolated poly-unsaturated photoreceptor membrane phosphatidylserine fraction does not strongly immobilize in the presence of Ca^{2+}. Fluorescence studies also do not exhibit any evidence for lateral phase-separation upon addition of Ca^{2+} to the total photoreceptor membrane lipids [29]. Bilayer destabilization in these lipids seems therefore to follow a less simple pathway, which might be relevant to physiological lipid perturbation processes where Ca^{2+} is involved, like membrane fusion during exocytosis.

The following aspects out of our results may shed some light on the underlying mechanism:

1) Maximal destabilization requires a Ca^{2+} to phosphatidylserine molar ratio of at least one. Under our experimental conditions at least 75% of the phosphatidylserine is then complexed to Ca^{2+}, assuming an apparent K_d of 1mM [31,33]. Lower Ca^{2+} to phosphatidylserine ratios will produce lower complexation levels in an approximately linear ratio, and at the same time produce an equivalent fractional conversion of bilayer to the hexagonal H_{II} phase (Fig. 3). It thus appears that every phosphatidylserine molecule independently contributes to the bilayer stabilization and that this effect is lost upon interaction with Ca^{2+} in an uncooperative fashion.

2) At maximum conversion the ^{31}P-NMR lineshape indicates a mixture of two phases with an intensity distribution of 85-90% in the hexagonal H_{II} phase and 10-15% in the bilayer phase. The linewidth of the latter cannot be exactly determined but is relatively large (at least 50 ppm) and thus would rather reflect phosphatidylserine (CSA = 54 ppm) than phosphatidylethanolamine or phosphatidylcholine (CSA = 38 and 45 ppm respectively) [35]. This distribution is maintained even at a large molar excess of Ca^{2+} (5-10 per phosphatidylserine). Taking the phospholipid composition of the photoreceptor membrane into account (18% phosphatidylserine, 42% phosphatidylethanolamine, 37% phosphatidylcholine), this strongly suggests that phosphatidylethanolamine together with the majority of the phosphatidylcholine enters into the hexagonal H_{II} phase and that calcium-phosphatidylserine remains behind in a fluid lamellar phase. This is in agreement with the behaviour of the purified phosphatidylserine fraction in the presence of Ca^{2+} and with the evidence available from model systems that phosphatidylserine at physiological

pH does not easily adopt in the molecular shape required for the hexagonal H_{II} phase, whether or not complexed to Ca^{2+} [24]. On the other hand, evidence has been presented that phosphatidylcholine is able to cotransfer with phosphatidylethanolamine into hexagonal H_{II} arrangements [26].

3) At fractional occupation of the phosphatidylserine molecules with Ca^{2+} at 25 ^{o}C, fractional conversion of bilayer to hexagonal H_{II} phase is observed (Fig. 2,3). Under those conditions an increase in temperature leads to gradually more bilayer destabilization. However now predominantly intermediate structures like lipid particles are generated, reflected in an isotropic lineshape, rather than additional hexagonal H_{II} phase [9,10]. Possibly the non-bilayer phase, already formed, now facilitates further perturbation of the lamellar phase [36], but the remaining uncomplexed phosphatidylserine only allows generation of intermediate structures like lipidic particles and no complete conversion to the undisturbed hexagonal H_{II} phase.

The picture emerges that bilayer stabilization by phosphatidylserine might involve direct electrostatic headgroup interaction with phosphatidylethanolamine, which is broken by formation of a calcium-phosphatidylserine complex. If the resulting bilayer destabilization is restricted to the intermediate environment of this complex, the fractional Ca^{2+}-effect can be explained. Additional experimental and theoretical analysis is required to examine this view in more detail. This will include investigation, by ^{31}P- and ^{2}H-NMR and differential scanning calorimetry, of the phase behaviour of phosphatidylserine/phosphatidylethanolamine mixtures of varying composition in dependence of Ca^{2+} occupation as well as of lipid modification

Physiological relevance of the polymorphism of photoreceptor membrane lipids

Dietary studies on animals involving depletion of essential fatty acids, indicate that poly-unsaturated acids are essential for correct functioning of the rod photoreceptor cell [37]. Although it has been demonstrated that under non-physiological conditions (freezing [38], and dehydration [36]) the intact photoreceptor membrane indeed exhibits polymorphic phase behaviour, all experimental evidence available so far indicates that under physiological conditions no other configuration for the lipid matrix than the bilayer is detectable. This is irrespective of whether it concerns isolated photoreceptor

membrane [9,10,11,17] or intact rod outer segments [35] and their dark/light history [9,10]. Light induced transient local bilayer perturbations may escape detection and cannot yet be completely excluded. However, it seems on basis of the present evidence illogical to suggest that the photoreceptor function requires a high degree of unsaturation of the photoreceptor membrane lipid matrix, because of the fact that such a high degree of unsaturation more easily allows conversion to non-bilayer phases than a less unsaturated matrix. Rather, the available evidence suggests that the adult rod outer segment takes every precaution not to perturb the photoreceptor membrane bilayer (strong bilayer stabilization by rhodopsin [33]; presence of intercalating cytoskeletal elements [39]) since perturbation would disturb the delicate structure of the stack of photoreceptor membranes and interfere with lateral protein diffusion and with the light-induced transmitter- and ion- fluxes.

At present two alternative reasons for the high degree of unsaturation of these photoreceptor membrane phospholipids are conceivable:

1) Newly synthesized photoreceptor membrane elements are inserted at specific sites of the rod outer segment [14,15,40,41] in a continuous very active process of membrane biogenesis. Possibly this process requires the polymorphic properties of poly-unsaturated phospholipids for smooth operation throughout life. Indeed, perturbation of the phospholipid metabolism results in erroneous membrane formation leading to a distorted outer segment structure and eventually to degeneration of the outer segment and loss of vision [42]. However, it has not been established whether this physiological deficit is accompanied by changes in lipid composition or in the degree of acyl chain unsaturation.

2) Phototransduction in the rod cell is a very efficient, rapid and regulable process [43], involving amoung other things a very dynamic protein-protein signal relay system and highly controlled Ca^{2+} and Na^{+} fluxes. These elements have contradictory requirements. A high rate of translational and rotational diffusion of intrinsic and extrinsic membrane proteins asks for a fluid unperturbed lipid bilayer and possibly specific lipid-protein interactions involving phosphatidylserine and/or phosphatidylethanolamine [44]. The specific ion fluxes require a very sealed membrane and a large store of Ca^{2+} ions inside the disk (ca. 100 mM "bulk" concentration). It is conceivable that in order to satisfy both requirements poly-unsaturated acyl chains are necessary since they are able to maintain a fluid matrix in the presence of high concentrations of Ca^{2+} ions

(intradiskal) or Mg^{2+} ions (intracellular). Furthermore, 22:6 3 fatty acyl chains have a high lateral and longitudinal flexibility and produce a membrane matrix with good sealing properties and relative little shear. These very aspects however also strongly promote the tendency to adopt non-bilayer configurations. Since this apparently would be catastrophic for the structure and function of the rod outer segment, a strong bilayer stabilizing factor needs to be incorporated into the photoreceptor membrane. The most efficient way to solve this structural problem then indeed is to use the visual pigment rhodopsin itself, being present in high density, for bilayer stabilization as well. This element will be addressed in more detail elsewhere [33].

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CHAPTER 5

MODULATION OF THE POLYMORPHIC PHASE-BEHAVIOUR OF BOVINE ROD PHOTORECEPTOR MEMBRANE LIPIDS: BILAYER STABILIZATION BY (RHOD)OPSIN.

L.C.P.J.Mollevanger, J. Olive and W.J. De Grip

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The photoreceptor membrane is the primary site of dim-light reception in the vertebrate eye. The highly specialized task of this membrane is reflected by its special composition. Over 90% of the membrane protein population is accounted for by the visual pigment rhodopsin [1], a membrane protein with a molecular weight of 41 kDa [2]. For its function as a photoreceptor rhodopsin has a strong absorption band peaking at 500 nm. This is generated by the interaction of the apoprotein opsin with a chromophoric group, 11-cis retinal, which is covalently bound to Lys-296. Following absorption of a photon, this chromophore isomerizes to the all trans form, inducing transient conformational changes in the protein [3]. Eventually, all-trans retinal leaves the protein moiety, resulting in the formation of the apoprotein opsin which can then be reactivated by recombining with 11-cis retinal.

Another unusual, but functionally relevant, feature of the photoreceptor membrane is its high content of polyunsaturated lipids. About 55% of the phospholipid acyl chains contains from 4 up to 6 unsaturated bonds [4,5]. Such a high degree of unsaturation is expected to enhance polymorphic phase behaviour of lipids in an aqueous environment: Formation of the hexagonal H_{II} phase in addition to lamellar bilayer structures or intermediate structures like "lipidic particles" [6,7,8]. Indeed it has been established that in dispersions of total lipids extracted from photoreceptor membranes, formation of non-bilayer lipid organization can be induced at temperatures over 20 ^{o}C by addition of divalent cations like Ca^{2+} or Mg^{2+} [9,10]. However it was simultaneuosly observed that under similar conditions the photoreceptor membrane itself does not show any indication for other lipid organizations than the bilayer up to temperatures as high as 50 ^{o}C [9,10,11].

Hence we suggested that rhodopsin, being the predominant membrane protein, might exert a stabilizing effect on the lamellar organization of its lipid environment. Here, we present further evidence supporting a decisive role of rhodopsin in stabilizing the bilayer configuration of the photoreceptor membrane. To that extent, we studied the following factors which could contribute to the observed stabilization in more detail : 1. The possible influence of rhodopsin on the interaction of Ca^{2+} ions with the photoreceptor membrane lipids. 2. The requirement of an intact tertiary protein structure. 3. The molecular range over which rhodopsin is able to

exert its stabilizing influence.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade.

Isolation of bovine rod outer segments

Fresh bovine eyes were obtained at the local slaughterhouse. The retinas were removed within 3 hours after the death of the animal. Rod outer segments (ROS) were isolated in dim red light by means of sucrose density centrifugation as described before [12]. When preparations of the apoprotein opsin were required the isolation was performed in the light, and NADPH was added to all buffers to convert retinal into retinol [12].

Regeneration of rhodopsin

In vitro regeneration of the apoprotein opsin to the photopigment rhodopsin was accomplished by addition of excess 11-cis retinal as a concentrated ethanol solution to rod outer segment opsin membranes resuspended in aqueous buffer, following the method described in [13].

Lipid extraction

Rod outer segments were washed twice with distilled water and centrifuged (5 ^{o}C, 80.000xg, 30 min). Total lipid extracts were obtained from these washed membranes by a modified Folch extraction [14]. All manipulations were carried out under a nitrogen atmosphere to prevent oxidation of the polyunsaturated fatty acids [4]. Lipid extracts were routinely analyzed for purity and composition by two dimensional thin layer chromatography and phosphate analysis (phospholipid composition), and GLC (fatty acid composition) following standard procedures [14,15]. The lipid extracts were stored under Argon at -80 ^{o}C.

Thermal stability of (rhod)opsin

The thermal stability at 60, 65 and 70 ^{o}C of rhodopsin in the photoreceptor membranes was monitored spectrophoto- metrically at 500 nm. The decrease in the absorption at 500 nm as a function of time was taken as a direct measure for the rate of protein denaturation. It was determined on aliquots taken after various time intervals by cooling down and solubilizing in detergent, as described before [13]. For opsin the decrease in the capacity to regenerate with 11-cis retinal under formation of rhodopsin was taken as a measure for thermal denaturation. In this case the regeneration capacity of

opsin samples, which were incubated at 50 and 55 °C for increasing time periods, was estimated by the increase of the 500 nm absorption by subsequent incubation at 25 °C with 11-cis retinal [13]. In both cases, the protein concentration, ca. 200 nmol (rhod)opsin per ml, was comparable to those used in the NMR samples. All measurements were carried out in a buffer containing 20 mM Mops, 10 mM $CaCl_2$, pH=7.2.

Preparation of recombinants of rhodopsin with endogenous lipids

Vesicles with a molar ratio of phospholipid to rhodopsin up to 150:1 were obtained by a reconstitution procedure adapted from the detergent dilution method described by Jackson et al. [16]. This is a very rapid procedure and thus avoids damage to the polyunsaturated, oxidation-prone lipids due to long dialysis times. All manipulations were carried out under an Argon atmosphere. Photoreceptor membranes were washed three times with distilled water and solubilized in buffer solution containing nonylglucose as detergent (reconstitution buffer: 20 mM Pipes, 20 mM Nonylglucose, 5 mM EDTA, pH=6.5) to a final concentration of 30 nmol rhodopsin/ml and stored overnight at 10 °C. The resulting solution was then centrifuged (100.000xg, 2 h, 4 °C) to precipitate unsolubilized material, yielding a very small white pellet mainly containing cytoskeletal proteins [Kamps and De Grip, unpublished results]. The supernatant was carefully removed and stored in ice under Argon.

Total lipid extracts from photoreceptor membranes were exposed to high vacuum for 2 hours at room temperature to remove all organic solvent. The residual lipid film was solubilized under Argon in ice-cold reconstitution buffer to a final concentration of 2 mM. The required volume of lipid solution was then added to the cooled photoreceptor membrane solution in order to obtain the required lipid/rhodopsin ratio. Due to limitations in the procedure (see below), the phospholipid/rhodopsin ratio in the finally obtained vesicles is however only maximally 75% of the initial ratio in solution.

The resulting solution is stored for 3 hours at 10 °C in order to ensure a homogeneous distribution of lipids over all micelles. Subsequently the solution is added dropwise under rapidly stirring to an excess of Pipes buffer (20 mM Pipes, 5 mM EDTA, pH= 6.5). The volume of Pipes buffer to be added is calculated to give a final volume in which the nonylglucose concentration is below the CMC (6.5 mM). The resulting vesicle suspension is centrifuged at 100.000xg for 16 hours. The pellet is washed 2 times with Pipes buffer to remove any residual detergent. The final vesicle-pellet is rapidly frozen and stored at -70 °C under Argon until further use.

The homogeneity of the vesicles with respect to lipid/protein ratio was checked by density gradient centrifugation on a linear 5-45% sucrose gradient prepared in three times diluted Pipes buffer. All bands containing light scattering material were collected separately and analyzed for rhodopsin and phospholipid content. Size and shape of the recombinant vesicles were analysed by thin section and freeze fracture electron microscopy as described below. The used reconstitution procedure which is based on very rapid dilution, produces a population of small unilamellar vesicles with a diameter between 50-80 nm. It usually gives only one well defined band upon sucrose density gradient centrifugation, in agreement with the results obtained by Jackson et al. [16] with octylglucose. Sometimes a small upper band was produced which however has the same protein and lipid composition as the major band, but probably contains a certain amount of detergent. A serious drawback of this procedure is the inverse relationship between the recovery and lipid/rhodopsin ratio. The recovery, based on rhodopsin, amounted to only approximately 60% for reconstitutions with a final rhodopsin/lipid ratio of 1:120 and was even worse (ca. 30%) for reconstitutions with a final rhodopsin/lipid ratio of 1:150. The recovery of lipid was still lower since the final rhodopsin/lipid ratio in the vesicles was maximally only 75% of the original ratio in detergent solution. Most of the unrecovered rhodopsin remained in the supernatant following centrifugation upon detergent dilution, since this still contained a light sensitive 500 nm absorption band. It consisted of material which did not sediment even after prolonged centrifugation at high speed, and probably represents very small mixed micelles. The exact nature of these remnants was not further investigated.

Unfortunately, the step-wise dilution procedure described before [17], which gives much better yields (> 80%), could not be employed for our present purpose, since it only allows preparation of homogeneous vesicles with a rhodopsin/phospholipid ratio of maximally 1/60-70 .

Determination of Ca^{2+} binding

Ca^{2+} binding to membranes was essayed on membrane or lipid vesicles depleted of multivalent cations by treatment with 15 mM EDTA. This reduces the endogenous Ca^{2+} content to below 0.3 mole of Ca^{2+} per mole of rhodopsin, or below 0.03 Ca^{2+} per phosphatidyl-serine [18]. Suspensions of ca. 400 nmol phospholipid/ml buffer (20 mM Mops, pH=7.2) were titrated at room temperature with a concentrated solution of $CaCl_2$ (up to 1 M), whereby the remaining free Ca^{2+} concentration was determined by potentiometry using a Ca^{2+}

selective electrode (Radiometer F2112 Ca) which gave a linear reading between $5*10^{-5}$ and 10^{-1} M Ca^{2+}. The performance of the electrode was independent of the presence of lipid vesicles, and was also not influenced by high concentrations of Mg^{2+}, Na^{+} or K^{+} ions (up to 150 mM). The electrode calibration was checked before and after each set of measurements. The Ca^{2+} binding equilibrium was reached instantaneously, since all readings were stable for at least 30 min after addition of an aliquot of calcium solution. The amount of bound Ca^{2+} was calculated from the amount of Ca^{2+} added and the free Ca^{2+} concentration obtained from the electrode reading.

^{31}P-NMR experiments

^{31}P-NMR spectra were recorded on a Bruker WM-200 spectrometer operating at 81 MHz. Measuring conditions were described before [10]. Broadband two-level ^{1}H decoupling was used with 0.3 Watt during the 0.9 sec interpulse-time, and 3 Watt during the 0.1 sec acquisition-time. Observation pulses were 15 µsec with a sweepwidth of 16 kHz. Temperature was controlled with a stream of thermostatted air with a precision of 1 ^{o}C. To improve the signal to noise in the spectra an exponential window was applied on the accumulated FID's before Fourier transformation which results in a 50 Hz linebroadening. Sample volume typically was 1.4 ml and NMR tubes of 10 mm diameter were used. All chemical shifts are relative to 80 % H_3PO_4.

Electron Microscopy

Electron microscopy is performed on samples fixed in darkness by addition of glutaraldehyde to a final concentration of 2%, and stored in the cold. For thin sectioning, samples were washed with buffer, postfixed in 1% OsO_4, dehydrated and embedded in Vestopal W. Thin sections are stained with uranyl acetate and lead citrate. For freeze-fracturing, small pellets of material are placed on gold discs, rapidly frozen in melting freon and stored in liquid nitrogen. Fracturing and replication is performed with a Balzers BA 301 at -150 ^{o}C. For the evaporation of platinum and carbon, an electron gun system is used. All preparations are examined in a Philips 400 electron microscope.

RESULTS

Rhodopsin does not affect Ca^{2+} binding to photoreceptor membrane lipids

Since millimolar concentrations of bivalent cations like Ca^{2+} and Mg^{2+} are required to induce polymorphic phase behaviour in photoreceptor

membrane lipid suspensions [9,10,11], we first investigated whether the bilayer stabilization of rhodopsin might be due to a significant decrease in affinity or total cation binding of the lipid matrix following incorporation of (rhod)opsin.

In order to quantify the binding of Ca^{2+} ions to either native photoreceptor membranes or vesicles containing total photoreceptor membrane lipids, Ca^{2+} titrations of suspensions of EDTA-treated membranes were followed by measuring the free Ca^{2+} concentration with a Ca^{2+} selective electrode. The amount of Ca^{2+} ions bound was calculated from the amount added and the resulting free Ca^{2+} concentration and expressed per mole of phosphatidylserine present. The total number of binding sites and dissociation constants were obtained from Scatchard plots, as shown in figure 1. The maximal amount of Ca^{2+} bound was not significantly different for these two preparations: 0.8 $\pm$ 0.1 (n=3) Ca^{2+}/phosphatidylserine for water washed native photoreceptor membranes, and 1.0 $\pm$ 0.1 (n=3) Ca^{2+}/phosphatidylserine for total photoreceptor membrane lipids. The apparent affinities were of the same order of magnitude : K_d = 1.1 $\pm$ 0.1 (mM) for the native photoreceptor membranes and K_d = 2.0 $\pm$ 0.1 (mM) for the total lipid extract. Exposure of the native membranes to light, causing conversion of

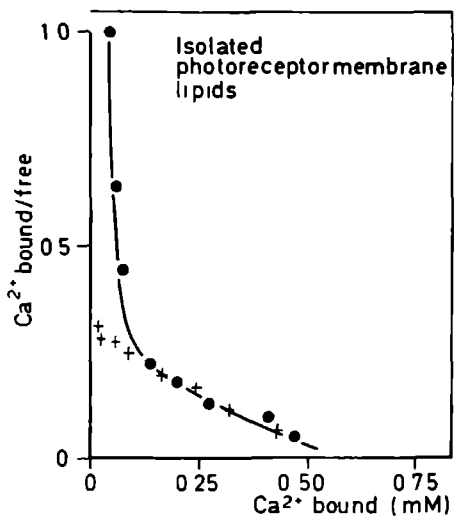


Figure 1 : Typical Scatchard plot of calcium binding by isolated photoreceptor membrane lipids, suspended in 20 mM Mops, pH 7.2 with (x) and without ( ) 100 mM KCl. The free calcium concentration was measured with a calcium-selective electrode and was varied between 10^{-5} M and 10^{-2} M. This particular sample contained 2.7 mg/ml phospholipid material, which is equivalent to a phosphatidylserine concentration of 0.6 mM. The "apparent high-affinity" sites represent surface charge effects since they "convert" to low-affinity sites in the presence of 100 mM KCl (x) or 100 mM NaCl (not shown). Since the latter conditions more resemble physiological conditions of ion strength, the high affinity binding

rhodopsin into opsin, did not lead to significant changes in these parameters with values for the maximal binding to opsin containing membranes of 1.0 ± 0.2 (n=2) Ca^{2+}/phosphatidylserine and for the K_d = 1.2 ± 0.2 (mM). Thermal denaturation of rhodopsin again did not produce significant changes in these properties. Finally, addition of Na^+ or K^+ up to 100 mM also did not markedly change the values for the K_d and total binding. Analysis of the binding curves by means of the Hill plot yields a linear relationship with n = 1.2 ± 0.1 (n=4). This suggests that Ca^{2+} interacts non-cooperatively in a 1:1 ratio with its binding site.

Thermal denaturation studies : A native tertiairy (rhod)opsin structure is required for bilayer stabilization

Subsequently we investigated whether bilayer stabilization by (rhod)opsin requires an intact protein structure. Hereto the thermal stability and the polymorphic behaviour was determined for dark (rhodopsin) as well as fully bleached (opsin) photoreceptor membranes under similar conditions (200 - 400 nmol (rhod)opsin/ml 20 mM Mops, 10 mM $CaCl_2$, pH = 7.2). The thermal stability was essayed spectrophotometrically by the decrease in intrinsic 500 nm absorbance (rhodopsin) or regeneration capacity (opsin) in time. Lipid polymorphism was determined by ^{31}P NMR spectroscopy. As a further check on vesicle structure, polymorphism and protein aggregation, thin section and freeze fracture ultramicroscopy was performed on parallel samples.

Thermal stability of (rhod)opsin:

Water washed photoreceptor membranes were incubated in darkness at elevated temperatures (60, 65 and 70 ^{o}C). The 500 nm absorption, characteristic for native intact rhodopsin, was measured as a function of time. Figure 2A shows a graphical plot of the obtained results. Up to 60 ^{o}C there is no substantial decrease in the 500 nm absorption over a period of more than two hours, indicating that rhodopsin is practically stable up to this temperature. However at 65 ^{o}C a slow decrease is measurable ($t_{\frac{1}{2}}$ = 160 min). At 70 ^{o}C the rate of denaturation of rhodopsin is further increased, manifesting itself in a more rapid decrease in 500 nm absorption ($t_{\frac{1}{2}}$ = 40 min).

Fully bleached photoreceptor membranes were analysed in an analogous way. At defined intervals aliquots were taken, rapidly cooled down and incubated with 11 cis-retinal upon which the resulting 500 nm absorption was determined. The relation between the regenerating capacity of opsin and the incubation

time is shown in figure 2B for two temperatures. At 50 °C there is only a gradual loss of regeneration capacity, ($t_{\frac{1}{2}}$ = 240 min). At 55 °C however a rapid decrease is detected ($t_{\frac{1}{2}}$ = 45 min).

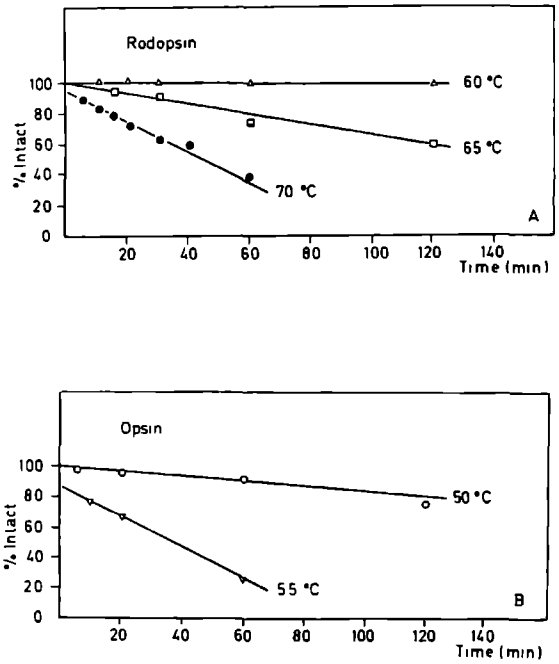


Figure 2 : A) Thermal stability of rhodopsin at temperatures of 60, 65 and 70 °C. The concentration of intact rhodopsin at each time-point was calculated from its 500 nm absorbance and normalized to the starting concentration, determined at 20 °C.
B) Thermal stability of opsin at temperatures of 50 and 55 °C. The concentration of intact opsin at each time-point was determined by its capacity to regenerate into rhodopsin upon subsequent addition of excess 11-cis retinal at 25 °C, measured as regenerated 500 nm absorbance. These values were normalized to control values taken at 25 °C. Since degeneration already starts during temperature equilibration, the curves with high degeneration rates extrapolate to 100% before time zero.

Lipid polymorphism:

^{31}P-NMR spectra of photoreceptor membranes were recorded at temperatures between 50 en 70 °C. For each sample a reference ^{31}P-NMR spectrum was recorded at 25 °C. At this temperature the ^{31}P-NMR spectra of all samples showed the typical lineshape that is consistent with phospholipids organized in a bilayer (not shown cf. ref. 10). Subsequently samples were incubated at elevated temperatures during 30 minutes for complete temperature equilibration before recording the ^{31}P-NMR spectrum. It takes about 45 min to record a spectrum with a sufficient signal to noise ratio. Figure 3 shows the ^{31}P-NMR spectra recorded at 55, 60, 65 and 70 °C for rhodopsin containing photoreceptor membranes. Up to 60 °C the spectra are similar to

those recorded at 25 ^{o}C, with a low field shoulder a high field peak and a chemical shift anisotropy of 45 ppm, showing that the phospholipids in the photoreceptor membrane are organized in a fluid bilayer. After 75 min at 60 ^{o}C (equilibration + recording) some change is noticable, involving only a minor percentage (10%) of the lipids which produce a broadened isotropic lineshape. Firstly at 65 ^{o}C a significant change in the lineshape of the spectrum is observed, involving about 40% of the lipids after 75 min incubation. The amount of the bilayer component decreases, and lineshapes representing non bilayer organization appear (i.e. lipids in a hexagonal H_{II} phase and micellar organization).

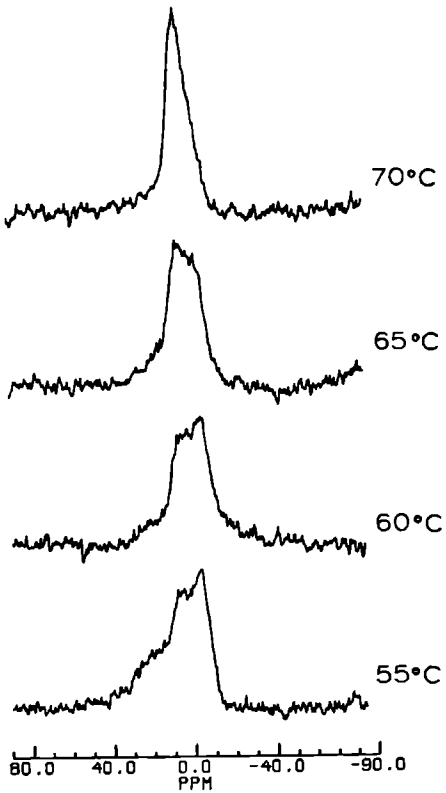
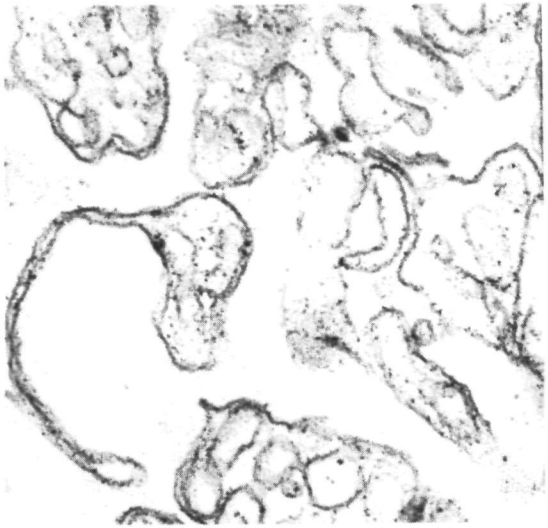


Figure 3 : ^{31}P-NMR spectra of water washed dark adapted photoreceptor membranes as a function of temperature. Before the measurement the sample was thermally equilibrated during 15 min. Each spectrum is an accumulation of 2500 FID's, and requires about 45 min. recording time. Samples typically contained 400-800 nmoles/ml of rhodopsin, and were suspended in : 20 mM Mops, pH 7.2, 10 mM Ca^{2+} in H_2O/D_2O (85:15, v/v).

At 70 °C the bilayer component in the spectrum has been strongly reduced after 75 min incubation. Instead the spectrum shows major components at -10 ppm and at 0 ppm representing lipids in a hexagonal H_{II} phase and lipids experiencing isotropic motion [19]. These temperature induced changes are not reversible. Upon recooling to 25 °C the spectra do not significantly change. Thin section and freeze fracture electron micrographs support this picture (Fig. 4). Before protein denaturation a liposomal vesicular structure is observed with diameters ranging from 750 to 1200 nm representing intact disk membranes. The freeze fracture morphology does not give any other evidence then phospholipids organized in a bilayer. After denaturation at 70 °C for 75 min the average diameter of the liposomes is considerably reduced (200 to 400 nm, Fig. 4C). This suggests that extensive intravesicular membrane fusion has occurred, resulting in a decrease in vesicle size. Freeze fracture micrographs indicate that protein denaturation does not cause extensive aggregation of rhodopsin molecules. However, patches with long tubular structures now appear, which are very reminiscent of lipids organized in a hexagonal H_{II} phase [20,21] (Fig. 4D).

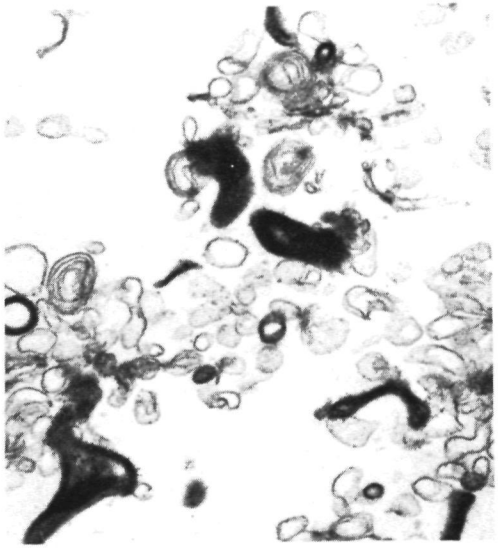
In order to ascertain that the observed phenomena are not due to thermal effects acting directly on the lipids themselves, the same set of experiments was performed on illuminated photoreceptor membranes, in view of the lower thermal stability of opsin as compared to rhodopsin. As shown above, irreversible loss of regeneration capacity of opsin already starts at temperatures over 50 °C, i.e. 15 °C below the onset of irreversible loss of the absorbance band of rhodopsin. Figures 5A and B show the ^{31}P NMR spectra of dark-adapted photoreceptor membranes at 45 and 55 °C. The lineshape is characteristic of phospholipids in extended bilayers. Thereupon the sample was fully bleached ,converting all rhodopsin to opsin. Subsequently, the ^{31}P NMR spectra in figure 5C and D were consecutively recorded. These latter spectra have dramatically changed and now mainly consist of a low field peak and a high field shoulder. The reversed asymmetry

Figure 4 : Electron micrographs of non-illuminated photoreceptor membranes incubated for 60 min. at 70 °C. Two parallel membrane samples were resuspended in : 20 mM Mops, pH 7.2, either without (A,B) or with 10 mM Ca^{2+} (C,D). After incubation the samples were processed for both thin section (A,C) and freeze fracture (B,D) electron microscopy as described in the method section. Bar represents 0.2µm.



A

B



C

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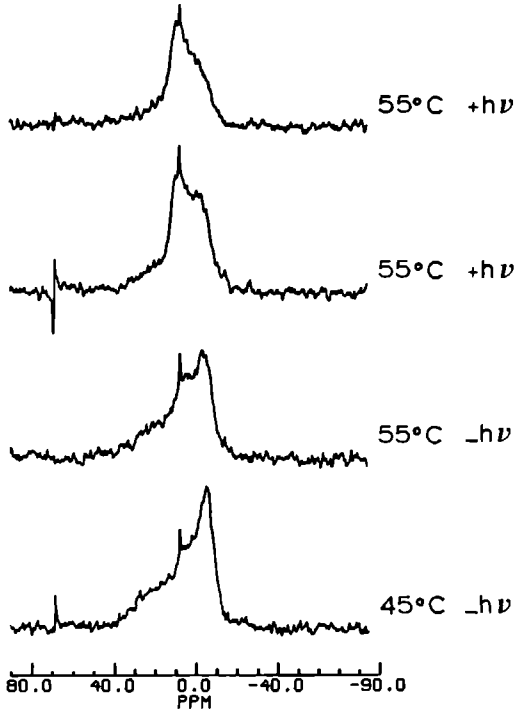

Figure 5 : ^{31}P-NMR spectra of water washed photoreceptor membranes as a function of temperature and illumination. Spectra of dark adapted photoreceptor membranes were recorded at 45 °C (A) and 55 °C (B). The same sample was then completely bleached at 55 °C and spectra were taken at 55 °C immediately after bleaching (C) or following one hour incubation at 55 °C after bleaching (D). Each spectrum is an accumulation of 2500 FID's, which requires about 45 min. recording time. Solution conditions: 400-800 nmoles/ml of rhodopsin suspended in ; 20 mM Mops, pH 7.2, 10 mM Ca^{2+} in H_2O/D_2O (85:15, v/v).

and the reduced chemical shift anisotropy, relative to the spectra in figures 5A and B, reflect a different phospholipid organization consisting of hexagonal H_{II} phase with some bilayer phase and isotropically moving lipids.

Again changes in the spectra observed upon protein denaturation are not reversed by lowering the temperature. Electron micrographs (Fig. 6A,B) show similar features as observed for unbleached photoreceptor membranes at 70 °C, (Fig. 4C and D): A reduction in vesicle size, and extensive presence of lipids organized in long tubular structures typical for the hexagonal H_{II} phase.

It is important to note that all changes observed by ^{31}P-NMR and ultramicroscopy to occur upon thermal denaturation are strictly dependent on the presence of Ca^{2+} ions. In the absence of Ca^{2+}, no or only limited (at

70 °C) conversion of large bilayer vesicles into a mixture of smaller vesicles, hexagonal H_{II} phase and "lipidic" particle is detected. (cf. Fig. 4 A,B vs. 4 C,D).

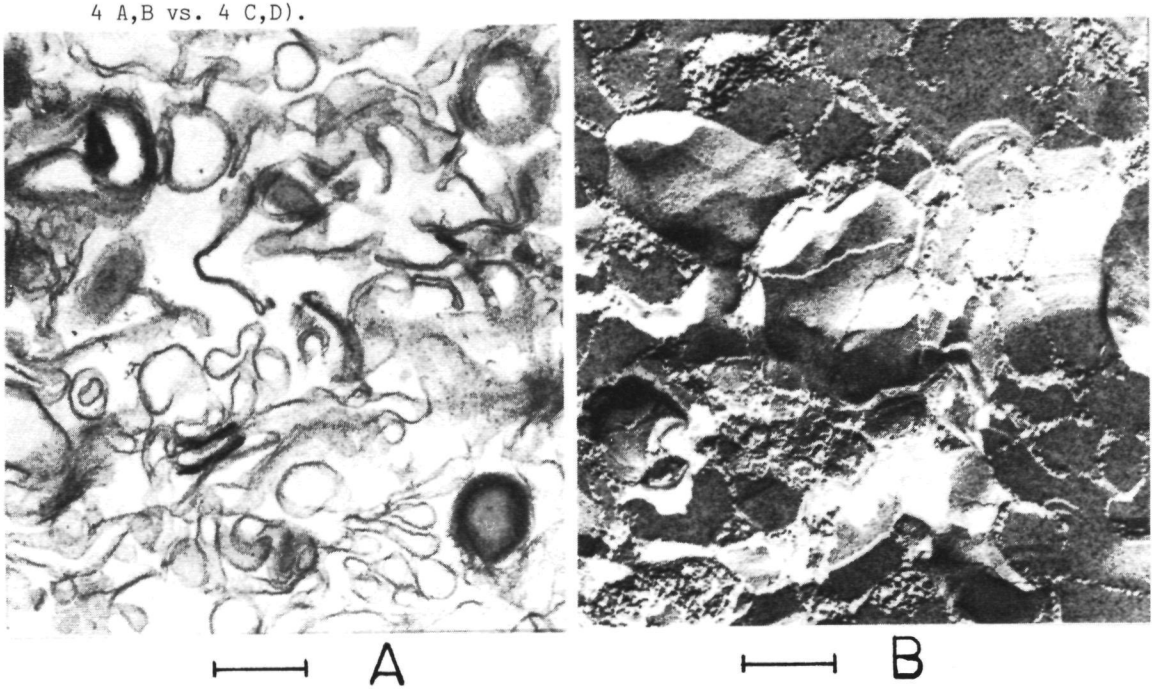

Figure 6 : Electron micrographs of totally bleached photoreceptor membranes suspended in : 20 mM Mops, pH 7.2, 10 mM Ca^{2+}, after incubation at 55 °C during 60 min. A) Thin section, B) Freeze fracture. Bar represents 0.2µm.

Recombinant vesicles : how far laterally extends the bilayer stabilization of rhodopsin.

Recombinant vesicles were prepared with a lipid/rhodopsin ratio about 2 times the natural ratio (120:1 and 150:1). The employed reconstitution procedure produces very small unilamellar vesicles with a mean diameter typically less then 100 nm (Fig. 7A). Higher ratios could not be obtained in the required large amounts due to to extensive loss of lipid material with this procedure. The ^{31}P-NMR spectra of both vesicle preparations indeed show a relatively sharp signal around 0 ppm (Fig. 8A), indicating phospholipid molecules undergoing rapid isotropic motion, due to the small size of the vesicles. Raising the temperature up to 45 °C did not change the shape of the ^{31}P-NMR spectra. No indication for phospholipids organized in the hexagonal H_{II} phase was found (Fig. 8A,B). In freeze fracture micrographs

non-lamellar lipid phase could also not be detected (not shown).

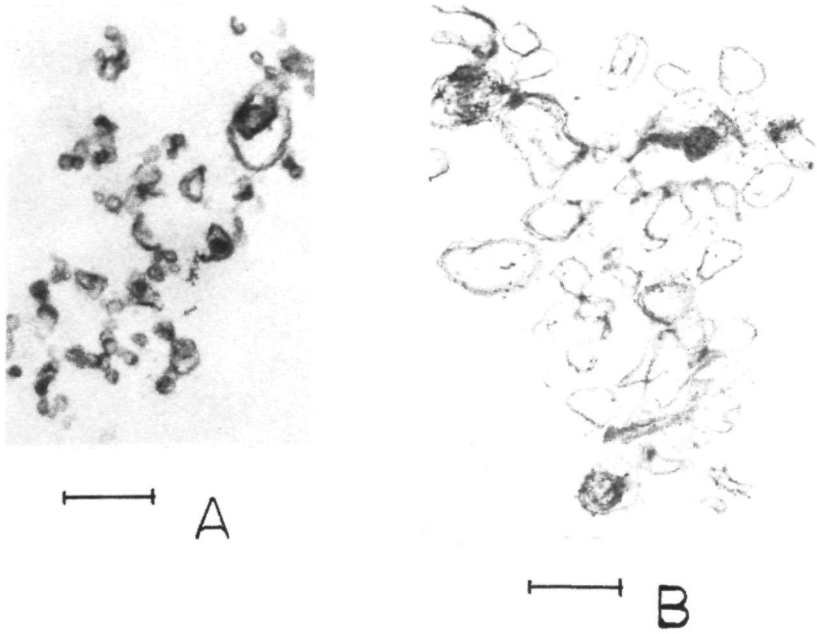

Figure 7 : Thin section electron micrographs of recombinant membranes of rhodopsin in photoreceptor membrane lipids with a rhodopsin-lipid ratio of 1:120 obtained by means of the rapid detergent dilution technique described in the method section. A) Suspended in 20 mm Mops, pH 7.2 at 25 ^{o}C, B) Sample identical to (A) but now after addition of 10 mM Ca^{2+} followed one hour incubation at 55 ^{o}. Bar represents 0.2 µm.

Subsequently addition of Ca^{2+} ions to these recombinant vesicles in a 1.5 molar excess to phosphatidylserine produced significant changes. Extensive vesicle fusion occurred upon raising the temperature, resulting in considerable increase in the vesicle diameter of both samples. This could be demonstrated both by ^{31}P-NMR spectrometry (Fig. 8C,E) and electron microscopy (Fig. 7B). The original narrow isotropic ^{31}P-NMR lineshape is broadened and changes to a lineshape characteristic for phospholipids organized in extended bilayers (low field shoulder and a high field peak, chemical shift anisotropy of 45 ppm). This is typical of vesicles with diameters over 250 nm. Addition of EDTA does not reverse these changes, indicating that they are not produced by mere aggregation of small vesicles. Indeed electron micrographs predominantly show larger monolamellar vesicles (300-400 nm diameter, Fig. 7B). The particle size is similar to that found in

photoreceptor membranes (cf. Fig. 4B). The particle density is however about two-fold lower, in agreement with the higher lipid/rhodopsin ratio. No other lipid configuration then the lamellar phase was detectable.

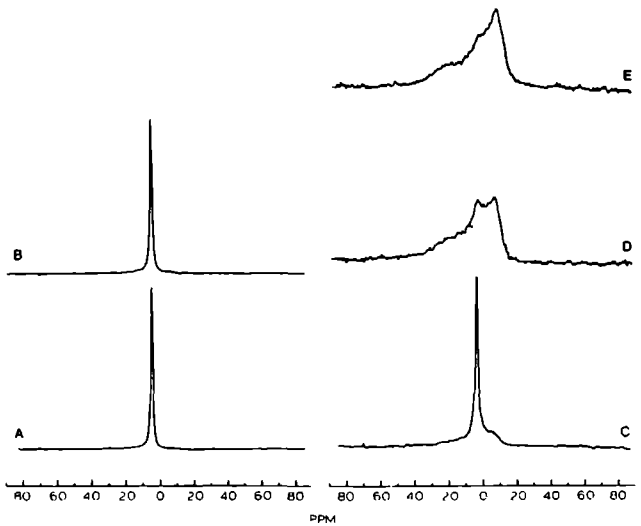

Figure 8 : ^{31}P-NMR spectra of recombinant membranes of rhodopsin in photoreceptor membrane lipids with a rhodopsin/lipid ratio 1:120, obtained by means of the rapid detergent dilution technique described in the method section. Samples are resuspended in 20 mM Mops, pH 7.2. Spectra were obtained after one hour incubation at 25 ^{o}C (A), and 45 ^{o}C (B). The sample was then recooled to 25 ^{o}C and Ca^{2+} added to a final Ca^{2+}/phosphatidylserine ratio of 2,5:1. Subsequent spectra were obtained after 1 hr incubation at at 25 ^{o}C (C), 35 ^{o}C (D) and 45 ^{o}C (E). In the spectra (D) and (E) the vertical scale has been increased by a factor of 4, compared to the spectra (A) to (C).

DISCUSSION

(Rhod)opsin stabilizes lipids in a bilayer configuration.

Recently it has been reported that isolated photoreceptor membrane lipids show polymorphic phase behaviour induced by divalent cations like Ca^{2+} and Mg^{2+} in millimolar concentrations, under physiological conditions of temperature, pH, and ionic strength [9,10]. Yet under identical conditions the lipids in the "natural" photoreceptor membrane do not show any polymorphic

behaviour. A possible role for rhodopsin in maintaining bilayer structure in the photoreceptor membrane was proposed. Several mechanisms by which a membrane protein like rhodopsin might influence the phase behaviour of its lipid environment were suggested, like a rhodopsin induced reduction in the affinity of the photoreceptor membrane lipids for Ca^{2+} ions or an inhibitory effect of protein structure or protein-lipid interaction on formation of non bilayer structures.

The present results lend further support to the concept that rhodopsin is the bilayer stabilizing factor in the photoreceptor membrane. Recombinant membranes consisting of rhodopsin, as the sole protein, and photoreceptor membrane lipids in a phospholipid /protein ratio up to 2.5 times the in vivo ratio in the rod disk membranes were prepared and characterized. They only showed lipids organized in the lamellar phase at temperatures as high as 45^{o} C, under conditions where the lipids without protein already convert to the hexagonal phase at 25^{o} C [9,10]. Remarkably, these recombinant vesicles do undergo fusion in the presence of Ca^{2+} ions at temperatures over 25^{o} C. The rate of this process seems to increase with higher lipid/protein ratios. Apparently under these conditions local disorder or structural defects, required to initiate the fusion process [22], are sufficiently present in the lamellar organization of these vesicles. But the presence of rhodopsin prevents that local disorder or defects spread over larger areas and convert the lamellar phase into a "lipidic particle" or hexagonal H_{II} phase.

Subsequently we investigated whether this bilayer-protection of rhodopsin is produced by affecting the binding of bivalent cations to the lipids or by a structural effect of the protein.

Bilayer-stabilization is not related to cation-binding.

In earlier work Hendriks et al. [18] investigated the Ca^{2+} binding properties of osmotically shocked photoreceptor membranes by equilibrium dialysis at 4 ^{o}C and arrived at a small population of high affinity sites (0.3/rhodopsin, K_d= 10-40 uM) and a much larger population of low affinity sites (10-13 per rhodopsin, K_d= 0.3-1.6 mM depending on the monovalent cation concentration). The approach, we have described, i.e. titration with Ca^{2+} ions at 20 ^{o}C using a Ca^{2+} specific electrode which measures the remaining free Ca^{2+} concentration, is rapid, more versatile and more suitable for pure lipid dispersions. The results obtained with this method agree very well with regard to number and affinity of the low affinity sites

(0.8 ± 0.1 /phosphatidyl serine, i.e. 9.6 ± 1 rhodopsin, K_d = 1.1 ± 0.1 mM). Values for apparent high affinity sites however show considerable scatter (n = 0.5 to 1.2 per phosphatidylserine). In the presence of 100 mM KCl or NaCl these sites however disappear (Fig. 1). Hence it is very likely that this "apparent high-affinity binding" does not represent discrete binding sites, but probably reflects surface charge effects. The high-affinity binding is therefore not further taken into account. We do not find significant dependance of the total number of binding sites or the low affinity K_d on the presence of Na^+ or K^+ up to 100 mM. Presently it is not clear whether this discrepancy with equilibrium dialysis may be due to thermodynamic and/or kinetic effects (temperature, incubation time). More importantly, we find no significant changes in the Ca^{2+} binding following either illumination or thermal denaturation of rhodopsin (n = 1.0 ± 0.2 /phosphatidyl-serine, K_d = 1.2 ± 0.2 mM). Most importantly, the isolated lipids have the same number of binding sites (1.0 ± 0.1 /phosphatidyl-serine) and an affinity (K_d = 2.0 ± 0.1 mM) which statistically is significant different, but physiologically insignificantly different from the intact photoreceptor membrane.

If the presence of (rhod)opsin would prevent the formation of non bilayer phases by interfering with the Ca^{2+} binding properties of the lipids, one would expect for the photoreceptor membrane either an at least ten-fold reduction in affinity (K_d > 20 mM), or an at least five-fold reduction in number of binding sites (n < 0.2/phosphatidylserine), since at this ratio of occupation already non-bilayer phases become apparent (Fig. 3, ref. 10). Both conditions are not fulfilled, so we have to conclude, that rhodopsin does not markedly interfere with Ca^{2+} binding by the photoreceptor membrane lipids, but does prevent subsequent conversion of the lamellar lipid phase into non-bilayer organizations.

Native structural properties of rhodopsin are involved in bilayer stabilization

At physiological temperature both rhodopsin, retinyl-opsin and opsin are able to stabilize the lipid bilayer [11]. Apparently stabilization does not require the presence of the chromophore. An early study by Hubbard [23] demonstrated that thermal denaturation of opsin became significant at temperatures over 50° C, i.e. about 15° C lower than the threshold for rhodopsin. This differential thermal behaviour of two structurally very

similar states of the visual pigment provides the opportunity to investigate whether a native tertiary protein structure is involved in bilayer stabilization. It allows to discriminate between temperature dependent changes in the lipid organization in the photoreceptor membrane caused by direct thermal effects on the lipids and changes coupled to alterations in the structure of rhodopsin. Hence we repeated and extended the studies of Hubbard under the conditions of our NMR experiments (ca. 200 nmol rhodopsin/ml, 20 mM Mops buffer, 10 mM $CaCl_2$, pH = 7.2). Slow thermal denaturation of opsin ($t_{\frac{1}{2}}$ > 240 min) was indeed observed at 50° C. The rate increased about five-fold at 55° C and at 60° C nearly complete denaturation was observed within 10 min. At this temperature, thermal denaturation of rhodopsin was however negligible. Rhodopsin denaturated slowly at 65° C ($t_{\frac{1}{2}}$ = 160 min), and the rate further increased at 70° C ($t_{\frac{1}{2}}$ = 40 min). This shows that under our NMR conditions we again observe a large difference in thermal stability of rhodopsin and opsin. Further independent evidence comes from a recent study using differential scanning calorimetry [24] where an irreversible peak of excess specific heat was observed between 52 and 60 ° C in illuminated photoreceptor membranes and between 65 and 74 ° C in rhodopsin containing photoreceptor membranes. These peaks should represent denaturation of opsin and rhodopsin, respectively.

When Ca^{2+} is present, membrane fusion processes begin to occur and non-bilayer resonances appear in the ^{31}P-NMR spectra simultaneous with thermal denaturation of (rhod)opsin. While the rate of loss of bilayer contribution to the spectra cannot really be determined due to the relatively long time required to obtain good quality spectra, there is a definite correlation with the rate of thermal denaturation. Since this correlation is: 1) observed for both rhodopsin and opsin, 2) there is a 15° C gap in thermal stability between these species, the appearance of non-bilayer phases cannot be due to direct thermotropic effects on the lipids, but must originate in a loss of the bilayer stabilization capacity of (rhod)opsin upon irreversible denaturation. This loss of bilayer stabilization capacity is not the result of extensive protein aggregation (which could considerably increase the effective lipid/protein ratio), since freeze fracture analysis does not indicate the formation of large isolated patches of particles. Hence, bilayer stabilization must reside in certain elements of the native (rhod)opsin structure which are lost upon denaturation. Since these elements only disappear during the final, irreversible, phase of denaturation, they probably reside in the intramembrane

domain of the protein. It is likely, that the structural stability of this domain largely determines the thermal stability of the protein, in view of its susceptibility to detergent action [13].

Physiological relevance

Up till now only a few membrane proteins, both extrinsic or intrinsic, have been studied with respect to their influence on lipid polymorphism [25]. Extrinsic membrane proteins like cytochrome C, cardiotoxin and the polypeptide gramicidine provoke formation of the hexagonal H_{II} phase [26,27,28]. Intrinsic membrane proteins like glycophorin, Ca^{2+}-ATPase and chlorophylase however stabilize the bilayer configuration [29,30,31,32]. From the results presented in this paper, together with results published previously [10], we conclude that rhodopsin follows the pattern of intrinsic membrane proteins. The exact range of this stabilization could not yet be determined because it proved yet technically difficult to obtain reconstituted vesicles with a lipid/rhodopsin ratio exceeding 150 : 1 in an acceptable yield. However we were able to prove that one rhodopsin molecule can efficiently stabilize at least 150 phospholipid molecules, i.e. two and a half times the ratio in the native photoreceptor membrane, under experimental conditions at which the extracted photoreceptor membrane lipids easily adopt non-bilayer phases [9,10]. This represents five to six molecular lipid shells per rhodopsin, a range which approaches that reported for the intrinsic membrane protein glycophorin [29]. It remains to be determined whether this stabilization is a property inherent to structural elements of membrane proteins (i.e. hydrophobic α-helical segments) or a specific property inserted into specific proteins.

From a physiological point of view it is easy to see that stabilization of the photoreceptor membrane lipids in the bilayer phase in the membrane in vivo is essential for a proper functioning of the rod photoreceptor cel. The regular stacked membrane arrangement observed in the rod outer segment is only possible with lipids organized in a stable bilayer. The question remains what physiological purpose serves the high degree of unsaturation of these lipids, which produces the unwanted tendency to adopt non-bilayer structures. This question will be adressed elsewhere.

ACKNOWLEDGEMENTS

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CHAPTER 6

^{31}P-NMR INVESTIGATION OF MAGNETICALLY ORIENTED ROD OUTER SEGMENTS

Leo C.P.J. Mollevanger, Edward A. Dratz, Ben De Kruijff, Cees W. Hilbers and Willem J. De Grip.

Eur. J. Biochem. 156 (1986), 383-390

The outer segment of the vertebrate rod photoreceptor cell has an elongated cylindrical structure [1] containing 500-2000 regularly stacked flat membrane disks, which are surrounded by a plasma membrane sheath (Fig.1). The lamellar disk structure is extremely regular, as indicated by X-ray diffraction studies [2], with an interdisk repeat distance of 29.5 $\pm$ 1.8 nm [3-5]. More than 85% of the total protein content of the disk membrane is accounted for by the visual pigment, the intrinsic membrane protein rhodopsin. For bovine rhodopsin a model with seven intrinsic helices has been proposed [6-9], which span the membrane and are oriented nèarly perpendicularly to the plane of the membrane [5, 10].

The regular stacking of the disk membranes results in a parallel disposition of the α helices of the 10^7- 10^9 rhodopsin molecules present per rod outer segment depending on species. These aligned helices provide the rod outer segment with a large overall diamagnetic anisotropy [11].

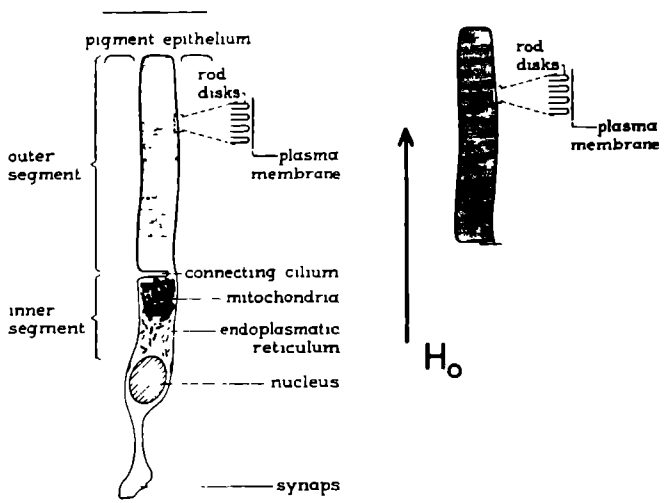

Figure 1 : Schematic drawing of a vertebrate rod photoreceptor cell (modified after Young [1]) showing the orientation of the outer segment when placed in a strong homogeneous magnetic field, with the field lines running parallel to the arrow.

Consequently intact rod outer segments in suspension orient themselves with their long axis parallel to the magnetic field in homogeneous magnetic fields as low as a few tenths of a Tesla [12]. Such oriented systems yield more refined information with orientation-sensitive analytical techniques like polarized light spectroscopy [13] and light scattering [14, 15]. The majority of ^{31}P-NMR studies, investigating the phospholipid organization and dynamic properties of lipids and membranes in aqueous suspensions, have been performed on randomly oriented samples, which give spectra of a rigid or a motionally averaged powder pattern [16]. However , a few ^{31}P-NMR studies on lipids and one study on biological membranes, which were partially oriented by coating on glass-plates, have been described [17-20]. Such oriented multilamellar systems appear to be very useful for studies of the mobility of the polar head-group region, the angular dependence of the chemical shift, the magnitude of the chemical shift anisotropy and the discrimination between various phospholipid head-group orientations and/or phase structures [17, 20].

The purpose of the present study is to present evidence that carefully isolated bovine rod outer segments retain their diamagnetic anisotropy and orient in the strong magnetic fields of modern NMR machines. ^{31}P-NMR spectra of oriented membranes can thus be obtained, which give much more specific information than non-oriented systems.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade . Ficoll 400 was obtained from pharmacia (Uppsala, Sweden), NMR tubes from Wilmad Inc. (Buena, NJ, USA). The "isolation buffer" contained 20 mM Mops (4-morpholinepropanesulfonic acid), 130 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, pH 7.2. The "NMR buffer" had the same composition as the isolation buffer, but contained in addition 20% v/v ^2H_2O, for deuterium lock of the NMR spectrometer, 0.02% NaN_3 as a bacteriostatic agent and 5% w/v sucrose, in order to increase the density of the medium and retard sedimentation of the rod outer segments during NMR measurements.

Isolation of rod outer segments

Bovine rod outer segments were purified under dim red light by means of a sucrose density gradient as described by De Grip et al. [21], or by means of a low-ionic-strength Ficoll/ sucrose density gradient [22], which is reported to

better preserve some functional aspects of rod outer segments [22] and hence may better maintain their morphology. Unless otherwise specified, 10 mM glucose was added to all buffers, since this was found to maintain higher concentrations of highenergy nucleotides like ATP and GTP (unpublished results). Briefly, the procedure was as follows. Bovine eyes were collected from the local slaughterhouse and dissected within 2 h after the death of the animal.Following gently homogenization of the retinas, the homogenate was filtered through a 125-mesh nylon gauze. The filtrate was layered on top of a continuous sucrose density gradient (23-36% w/w), or a Ficoll-400/sucrose gradient (600 mM sucrose + 5% w/w Ficoll to 20% w/w sucrose + 16% w/w Ficoll). Following centrifugation at 100,000 x g for 1.5 h at $7^{o}C$ the sharp band containing the rod outer segments [21, 22] was collected and diluted with one volume of isolation buffer. Subsequently a low-speed centrifugation step (3000 x g, 10 min, $4^{o}C$) was performed in order to obtain pellets containing the larger, more intact rod outer segments structures, while smaller fragments remained in suspension. The yield was 15-20 nmol rhodopsin/retina for the sucrose preparation and 10-15 nmol rhodopsin/retina for the Ficoll/sucrose preparation. The absorbance ratio A_{280}/A_{500} for these preparations was typically 2.4- 2.6. The opsin content [21] was less than 5%.

Sample preparation for NMR

The low-speed membrane pellets were carefully resuspended in 6 ml NMR buffer by gently spraying buffer over the soft pellets using an adjustable 1-ml pipette with plastic tip. After a second centrifugation step (3000 x g, 10 min, $4^{o}C$) the pellet was resuspended in the same buffer to a final concentration of 800 nmol rhodopsin/ml. Of this suspension 1.4 ml was transferred into a 10-mm NMR tube using a disposable pipette. Samples prepared in this way could be stored for more than 24 h at $4^{o}C$ in a refrigerator without noticeable loss of orienting capacity as measured by ^{31}P-NMR. The latter property was, however, largely destroyed by one freezing/thawing cycle.

Non-orienting membranes were obtained by hypotonic lysis of the intact rod outer segments with two water washings including a 100,000 x g, 30-min centrifugation step at $4^{o}C$. The resulting pellet of lysed rod outer segments was resuspended in the NMR buffer to a concentration similar to that described above.

NMR measurements

^{31}P-NMR measurements were routinely performed on a Bruker WM 200 spectrometer at 81 MHz with a dual ^{13}C-^{31}P, 10-mm probe, using 15 us

(60°) pulses, an interpulse delay of 1 s, and a sweepwidth of 16 kHz. Twolevel, high-power broadband ^{1}H decoupling (0.3 W between pulses and 3 W during data acquisition) was employed to remove ^{1}H-^{31}P dipolar coupling. Each spectrum is an accumulation of 2000-5000 transients. In order to increase the signal-to-noise ratio the accumulated free induction decays were exponentially filtered before Fourier transformation, resulting in a 50-Hz line broadening in the spectra shown in the figures. Spectra without line broadening did not show additional features except for a much lower signal-to-noise ratio and somewhat sharper peaks for the two metabolites near 0 ppm. All chemical shifts are given relative to external 80% H_3PO_4.

Some ^{31}P-NMR spectra were obtained at 36.4 MHz on a Bruker WH 90 with measuring conditions identical to those used in [23] or at 202 MHz on a Bruker WH-500 with the same measuring conditions used at 81 MHz, except for 5 W decoupling during data acquisition.

Sample temperature in all spectrometers was regulated within $\pm$ 1°C by a stream of thermostatted air or nitrogen. T_1 measurements were carried out at 81 MHz using the inversion recovery technique with an interpulse time of 5 sec (approx. 5 x T_1) to allow near complete relaxation of the ^{31}P spins [24].

RESULTS

Preparation of rod outer segments

According to Chalazonitis et al. [12] morphologically intact frog rod outer segments orient with an efficiency higher than 90% in magnetic fields greater than 1 T (10 Kg). Efficient orientation of rod outer segments depends heavily on an intact regular disk stacking, therefore it is essential to isolate the rod outer segments as intact as possible. Comparison of the two isolation methods, described in Materials and Methods, yields the following conclusions. (A) The combined Ficoll-400/sucrose isolation procedure, which is reported to preserve cellular structures better than the pure sucrose procedure [22], did not result in preparations with better orientation in our hands. Since pure sucrose gradients are easier to prepare, more economic in use, and provide considerably higher yields, the sucrose procedure has been routinely used for the preparations described in this study. (B) Only fresh retinas can be used. Preparations from frozen retinas show very little or no orientation at all, indicating heavy structural damage. For this study retinas were removed from bovine eyes, maintained in the dark at room temperature,

within 3-4 h after enucleation. When the procedure and precautions given in "Materials and Methods" are followed with care, nine out of ten preparations preserve the same maximal ability to orient in a magnetic field.

Intact versus lysed rod outer segments

Figure 2 presents typical ^{31}P-NMR spectra obtained for intact (A) and hypotonically lysed (B) bovine rod outer segment preparations. Lysis results in large single-walled vesicles [21] and figure 2B indeed shows a typical ^{31}P-NMR spectrum of large vesicles containing phospholipids in a bilayer arrangement: a broad (45 ppm) resonance with a high-field peak at -10 ppm.

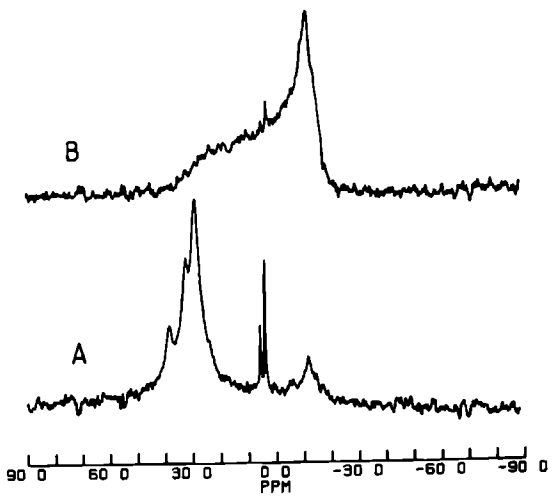

Figure 2 : ^{31}P-NMR spectra (81 MHz) of an intact non-illuminated bovine rod outer segment preparation (sucrose procedure) suspended in NMR buffer (A) and the same preparation after two additional water washes and subsequent redispersion in NMR buffer (B). The sample contained material harvested from 60 bovine eyes with a final rhodopsin concentration of 800 nmol/ml. Spectra were taken at 25°C and each spectrum is an accumulation of 3000 free induction decays. For spectrometer settings see Materials and Methods.

Similar spectra have been reported before for photoreceptor membrane vesicles [23, 25-28]. The "intact" preparation, which shows a largely preserved disk stacking upon electron microscopic analysis [21], gives a totally different ^{31}P-NMR spectrum, however (Fig.2A): it shows a relatively sharp ($\Delta_{\frac{1}{2}}\nu$ 15 ppm) resonance at low field around 32 ppm, which contains 75-80% of the total spectral intensity and is composed of three partially overlapping resonances, peaking at 37 ppm, 31 ppm and 28 ppm. The rest of the spectrum,including the

relatively small peak at the highfield side of the spectrum (around -14ppm), contains 20-25% of the total intensity, excluding the sharp metabolite peaks. Although less pronounced than on the low-field resonance, this high-field peak also seems to contain fine structure, suggesting that it consists of more than one component. From more than ten independent spectra of different samples we conclude that the high-field peak also is a superposition of at least three partially overlapping peaks at -11.5 ppm, -14 ppm and -17 ppm (cf. Fig.6)

Intact rod outer segments are oriented in the magnetic field

Features of the spectrum in figure 2A are in agreement with the expected axial orientation of the rod outer segments with respect to the magnetic field. Under these conditions the plane of the disk membranes and the magnetic field lines are at right angles to one another and the phospholipid head-groups are therefore expected to give a relatively narrow (10-20 ppm) resonance around 25-30 ppm [18-20].

The following two experiments (I,II) provide evidence that the spectrum in figure 2A is indeed due to orientation.

I. ^{31}P-NMR spectra of intact preparations, taken at different field strengths (11.7 T, 4.7 T and 2.1 T, corresponding to 202 MHz, 81 MHz and 36.4 MHz respectively) are shown in figure 3A,B,C. The overall line-shape of the spectra is similar and the chemical shifts of the major peaks are not detectably field-strength-dependent. In the spectra taken at 2.1 T (Fig.3 C) there is much more intensity between the high and low-field peaks than at higher field and the intensity distribution has shifted significantly to the high-field peak (cf. also Fig.4 A versus C). We conclude that at the lower field strength the bovine rod outer segments, which are much smaller than the frog rod outer segments studied in [12], are not yet maximally oriented. Since the spectra obtained at 4.7 T and 11.7 T (Fig. 3B and A) do not differ significantly in these respects, maximal orientation of bovine rod outer segments has indeed been achieved at a field strength of 4.7 T. At the highest field strength investigated (11.7 T), the stronger ^{1}H-^{31}P coupling could, however, not be completely decoupled, leading to loss of resolution (Fig. 3A). Hence, all spectral analyses were performed on spectra recorded at 4.7 T.

II. Spectra $\pm$ spinning in the Bruker WH 90. In the 81 MHz and 202 Mhz superconducting spectrometers the spinning axis is parallel to H_o. However, in the 36 MHz spectrometer, the magnetic field runs perpendicular to the long axis of the sample tube. Therefore the 36 MHz-machine can simply discriminate

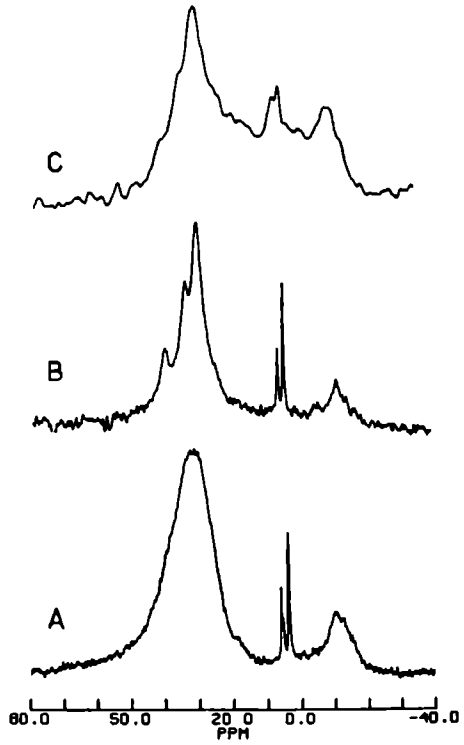

Figure 3 : ^{31}P-NMR spectra of intact bovine rod outer segments at 25°C, at different magnetic field strengths (11.7 T, 4.7 T and 2.1 T) corresponding to 202 MHz, 81 MHz and 36.4 MHz respectively. These spectra are recorded without spinning. (A) 202 MHz; (B) 81 MHz and (C) 36.4 MHz. Other conditions as under Figure 2.

between orientation and non-orientation by sample-tube rotation experiments using the same sample of intact rod outer segments. The rod outer segments orient at too slow a rate ($t_{\frac{1}{2}} \approx 5$ sec [29, 30]) to be able to compensate for spinning-induced loss of orientation. Hence, spinning at approximately 20 Hz should lead to a random distribution during the acquisition time (0.25 s) thereby converting an oriented spectrum in the non-spinning condition into a normal bilayer powder spectrum. This prediction is indeed borne out by experiment (Fig.4 A, B). When the sample tube is not spinning, orientation of the rod outer segments in the magnetic field is expected, and the resulting spectrum (Fig.4 A) indeed is similar to that in figure 2A. Spinning of the same sample randomizes the orientation and yields a perfect 'random bilayer' spectrum from intact rod outer segments (Fig.4 B),which is similar to that of the lysed preparation in figure 2B. The total resonance intensity is equal in figure 4A and B, demonstrating that no loss of specific phosphate resonances has occurred between figure 4A and B. In order to prove that the preparation was still intact and able to orient, the same sample was then transferred to

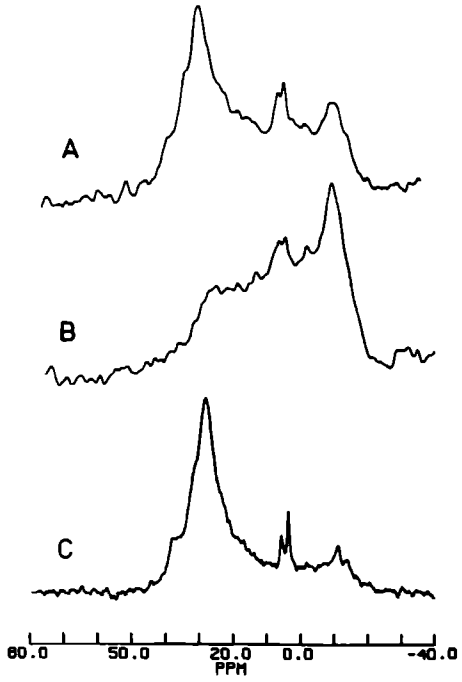

Figure 4 : ^{31}P-NMR spectra of the same sample of intact bovine rod outer segments taken consecutively at 36.4 MHz (A,B) and at 81 MHz (C). (A) Taken without spinning of the sample tube; (B) and (C) taken with spinning in the different machines. The sample is spun about an axis perpendicular (B) or parallel (C) to the magnetic field lines. (A) Spectrum at 36.4 MHz (Bruker WH 90) measured with a non-spinning sample tube. (B) Same sample measured with the same sample tube, but spinning at a rate of 20 Hz with the spinning axis perpendicular to the magnetic field. (C) Same sample, after measuring the spectrum in 4B now transferred to a Bruker WP 200, and measured at 81 MHz. Sample tube spinning at 20 Hz. Spinning axis parallel to the magnetic field. Other conditions as under Figure 2.

the Bruker WP 200, where the field lines run parallel to the long axis of the sample tube and spinning should not disturb the orientation. Figure 4C shows the same sample as figure 4A and B, now measured in the WP 200 under spinning conditions. A high-quality oriented spectrum is again obtained, which now is virtually identical, whether generated under spinning or non-spinning conditions.

Additional evidence for orientation is provided by the observations that the intact preparations are thermally rather labile. Upon incubation at 35^{o}C the oriented spectrum shows increasing contribution of the random bilayer spectrum. Observation under the light microscope reveals concomittant swelling, curling and deterioration of the outer segment structure under these conditions. Similar phenomena, both structurally and spectrally, can be induced by freezing/thawing or by hypotonic lysis of the intact preparations (cf.Fig. 2B versus A) Interestingly, the precense of a magnetic field enhances the structural stability of intact preparations, since samples held in the magnetic field loose their ability to orient at a lower rate than identical

samples held at the same temperature outside of the field.

These facts taken together present compelling evidence that ^{31}P-NMR spectra , like those shown in figures 2A, 3 and 4A,C represent magnetically oriented rod outer segments.

Analysis of the oriented spectrum

The isolation of intact well-orienting preparations is a matter of routine, when the given procedures are followed carefully. In addition, the temperature at which the spectra are recorded does not strongly influence their line-shape, as long as the conditions do not have a marked effect on the structural stability of the intact preparations. Spectra recorded between 5°C and 25°C do not show significant differences in line-shape or chemical shift. At 35°C the intact structure rapidly deteriorates (50% loss in major low-field peak within 4 h) and the spectra become increasingly dominated by a random bilayer line-shape (not shown). At temperatures below 10°C the orientation remains stable for at least 24 h.

Dominant features of the spectrum of oriented rod outer segments (cf. Figs 2A, 3B, 4C) are a large structured low-field resonance (+32 ppm), a smaller broad resonance peak at high field (-14 ppm) and two sharp resonances at 5.4 ppm and 3.5 ppm. The sharp resonances are thought to represent soluble phosphate-containing compounds. These resonances are still present after hypotonic lysis of intact rod outer segment preparations, but can be nearly completely removed by subsequent centrifugation and one additional buffer washing. The peak at higher field (3.5 ppm) coincides with added phosphate and hence presumably represents inorganic phosphate (P_i). In view of its chemical shift, the other sharp resonance is tentatively assigned to sugar phosphate and/or ethanolamine and choline phosphate esters. Upon incubation of intact preparations, the intensity of the latter peak remains fairly constant, but the P_i resonances increases in intensity with a temperature-dependent rate. The increase in free phosphate is probably due to a deficit in energy consumption versus recharge in these isolated cell-organelles, resulting in net hydrolysis of high-energy compounds liberating P_i. Nucleotide analysis of intact preparations demonstrates the presence of substantial amounts of nucleotide triphosphates and diphosphates. (In total up to one per rhodopsin; De Grip and Van Groningen, unpublished.) Corresponding resonances (α around -10 ppm, β around -20 ppm, γ around -5 ppm) can, however, usually not be detected above the noise level of the spectra. These peaks must be broadened

below detection by binding to obvious rod outer segment proteins (G protein, cyclase, kinases, ATPases ect.). The small feature in figure 2A at -5 ppm is not reproducibly observed and probably represents noise and not a nucleotide -phosphate resonance.

^{31}P-NMR spectra of liquid-crystalline structures, like membranes, reflect the distribution of orientation of the phospholipids with respect to the H_o field. In bilayer and hexagonal H_{II} phases this leads to typical line-shapes [31]. Since morphologically intact rod outer segments will align with their long axis parallel to the magnetic field, the major part of the membrane bilayer has its director parallel to H_o ($\sigma_{||}$). The two main resonances at +32 ppm and -14 ppm in the oriented spectrum must represent two phospholipid pools with different orientations with regard to the magnetic field. Phospholipids oriented parallel to, i.e. with their membrane plane perpendicular to, H_o will resonate near +30 ppm [18-20], which corresponds to the position of the major peak (75-80% of total intensity). The minor peak around -14 ppm corresponds to phospholipids oriented perpendicular ($\sigma_\perp$) to the field [20]. This peak accounts for 10-15% of the total intensity. The intensity ratio $\sigma_{||}$ to $\sigma_\perp$ (5-6:1) is a measure for the phospholipid distribution over these two pools.

The "parallel" peak is clearly split into three separate components and the shape of the "perpendicular" peak suggests a similar splitting. Since the chemical shift anisotropy of the major phospholipid classes in the rod outer segment membrane, phosphatidylethanolamine (PtdEth), phosphatidylcholine (PtdCho) and phosphatidylserine (PtdSer) [23,25,27], is significantly different, even under the axial symmetrical conditions of a bilayer [20], we considered that these separate resonances might represent different phospholipid classes. In other words we propose that the increased resolution due to orientation serves to resolve individual phospholipid classes as well as differently oriented phospholipid pools! The following arguments support this proposition. (A) We simulated the low-field major peak of the spectrum, using three Lorentzian lines with a chemical shift of 37 ppm, 31 ppm and 27 ppm, respectively, which were fitted to the experimental spectra by visual adjustment. The best fit, which was quite satisfactory (Fig.5), yields a percentage distribution of peak areas of 18%, 38% and 44%. These peaks appear at the appropriate positions and their areas correspond very closely to the phospholipid composition reported for the intact photoreceptor membrane (19% PtdSer, 38% PtdCho and 43% PtdEth; [27]). (B) The peaks at $\sigma_{||}$ can also be

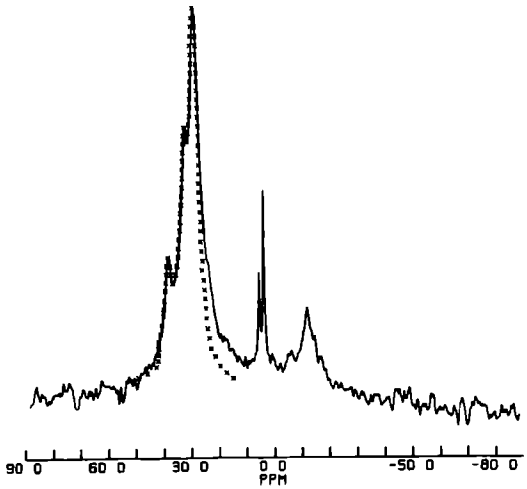

Figure 5 : Simulation of the low-field peak in ^{31}P-NMR spectra of oriented rod outer segments. The solid ^{31}P-NMR line (----) represents ^{31}P-NMR spectrum (81 MHz) of intact bovine rod outer segments, measured at 25°C. (xxxx) Simulation of low-field part of the ^{31}P-NMR spectrum. Simulation is based on three Lorentzian lines positioned at 37 ppm, 31 ppm and 27 ppm. Optimal fit by visual comparison requires a line-width of 260 Hz, 360 Hz and 320 Hz and a relative intensity of 18%, 38% and 44% respectively.

paired with the partially resolved components at $\sigma_\perp$ to yield $\Delta\sigma$ values, which are in good agreement with reported values for the chemical shift anisotropy of PtdSer, PtdCho and PtdEth respectively (Table 1). We conclude that the three resonances which generate the major peaks indeed represent the different phospholipid classes. Thus, the resonances at 37 ppm and -17 ppm are assigned to PtdSer, at 31 ppm and -14 ppm are assigned to PtdCho and at 27 ppm and -11 ppm are assigned to PtdEth. The high-field peak has the shape of the mirror image of the 30 ppm peak but, owing to the lower resolution of the separate resonances in the high-field peak and the fact that other structural elements also contribute to this area (see Discussion), we could not reliably decompose it into subpeaks.

The spin lattice relaxation time (T_1) was measured in the oriented rod outer segment preparations by the inversion recovery technique. The T_1 of the two main phospholipid pools at $\sigma_\parallel$ and $\sigma_\perp$ appears not to be significantly different: 1.2 s for both the parallel and the perpendicular orientation. Resolution was not good enough to determine the T_1 of the three separate resonances, i.e. the individual phospholipid classes. However, the partially

relaxed spectra do not provide any indication that T_1 values of the individual lipids are significantly different from the average value. As expected, the T_1 value of the soluble compounds is significantly higher (2.5 sec). However, also in the partially relaxed spectra no metabolite peaks below 0 ppm could be detected. For instance no indication for a metabolite resonance around -5 ppm appeared.

Finally the effect of illumination on the ^{31}P spectra of oriented samples was investigated. Since the accumulation of one spectrum with sufficient signal-to-noise ratio takes at least 30 min, no kinetic measurements were done. Instead, rhodopsin (unilluminated conditions), predominantly metarhodopsin I (illumination at 4°C) and metarhodopsin III + opsin (illumination at 20°C) were compared. No significant differences could be detected between the oriented spectra obtained under these three conditions, except that upon illumination the P_1 resonances shifted upfield by 0.3 ppm, which implies acidification with about 0.2 pH unit.

Table 1. Comparison of the literature values of the chemical shift anisotropy ($\Delta\sigma$) for the ^{31}P signal of the phospholipids PtdCho, PtdEth and PtdSer [30], and $\sigma_\perp$, $\sigma_{||}$ and corresponding $\Delta\sigma$ values for the components, observed in the ^{31}P-NMR spectra of oriented rod outer segments.

Species	^{31}P-NMR spectrum of rod outer segments			Corresponding values for pure phospholipid [30]
	$\sigma_{||}$	$\sigma_\perp$	$\Delta\sigma(\sigma_{||}-\sigma_\perp)$	$\Delta\sigma$
	ppm			
PtdSer	37	-17	54	55
PtdCho	31	-14	45	45
PtdEth	27	-11	38	41

General aspects of orientation

Thanks to a high degree of orientation of a membrane protein, which is present in high concentration, rod outer segments have a strong diamagnetic anisotropy [12, 30]. Worcester [11] concluded, from ultraviolet optical dichroism studies, that the net orientation of aromatic protein side chains was too small to contribute significantly to the diamagnetic anisotropy of rod outer segments. Instead, he presented quantitative arguments that the dominant diamagnetic anisotropy of the rod outer segment membrane orginates in the protein α helices. If the diamagnetic anisotropy of the lipid chains were to dominate, orientation of the rod axis perpendicular to the magnetic field would result [32, 33]. Thanks to their strong diamagnetic anisotropy, intact frog rod outer segments easily orient in magnetic fields over 1 T [12], and are one of the few naturel membranes, together with chloroplast grana [13], allowing orientation-dependent measurements. Magnetic orientation of rod outer segments has already been elegantly exploited in linear infrared dichroism studies [5] and orientation-dependent light-scattering studies [14] using externally applied magnetic fields.

In modern superconducting NMR spectrometers, operating at frequencies over 20 MHz for ^{31}P nuclei, the intrinsic magnetic field might be strong enough (> 1 T) to orient morphologically intact bovine rod outer segments. Our results present the following arguments that this is indeed the case for field strengths of at least 4.7 T. (A) Intact preparations yield ^{31}P-NMR spectra quite different from photoreceptor membrane vesicles. (B) The line-shape of the spectra is independent of field strength above 4.7 T. (C) The observed shifts (major resonance around 32 ppm = $\sigma_{||}$) are in accordance with orientation of the rod outer segments parallel to the magnetic field. (D) The individual phospholipid classes give relatively sharp ($\Delta\nu_{\frac{1}{2}} \approx 4$ ppm) resonance peaks close to the reported $\sigma_{||}$ positions of the pure lipids (PtdSer, 37 ppm; PtdCho, 31 ppm; PtdEth, 27 ppm). (E) Structural deterioration of intact preparations converts the oriented spectrum into a random bilayer spectrum. (F) Rotation of the sample in the plane of the magnetic field cancels out the magnetic orientation, again yielding a random bilayer spectrum.

Well-orienting intact bovine rod outer segment preparations are easily prepared by routine sucrose gradient procedures [21]. Addition of glucose to the isolation buffers better maintains the energy charge of these

preparations, which enhances their structural stability.

Correlation with morphological subelements

On the basis of the morphology of the rod outer segments, and our knowledge about the direction of its orientation in magnetic fields [12], we can try to attribute spectral features to morphological substructures (Fig.6). The flattened sides of the disk membranes, which, based on electron-microscopic morphometry, comprise about 85% of the total amount of rod outer segment membranes, have their phospholipids oriented parallel to the magnetic field and produce the major peak at 32 ppm. The rod outer segment plasma membrane phospholipids (ca. 6%) are largely oriented perpendicular to the field and will contribute to the minor peak at -14 ppm. The rim stuctures at the disk margin (ca. 10%) have a vesicular form and will produce a spectrum similar to a random bilayer, which contributes about half of its intensity to the minor peak and adds intensity to the middle part of the spectrum between 5 ppm and 20 ppm. Any non-orienting membrane material present will also produce

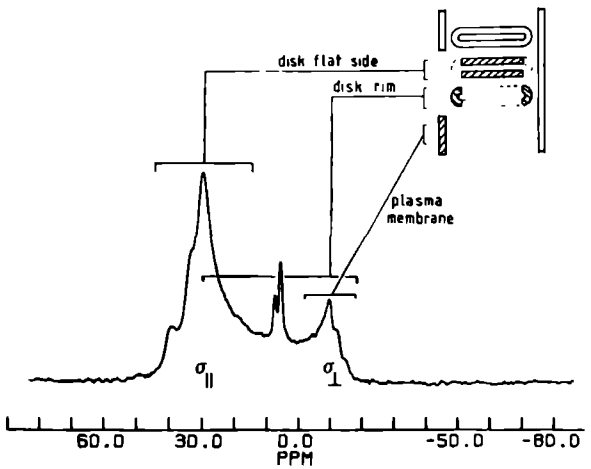

Figure 6 : Correlation between the peak distribution found in ^{31}P-NMR spectra of magnetically oriented bovine rod outer segments and their morphological substructures. The spectrum is a result of the addition of ten independent spectra. Thanks to the improved signal-to-noise ratio the substructures in the high-field peak are now more evident.

a random bilayer spectrum. The observed intensity distribution in the oriented spectra (75-80% of the spectral intensity downfield from 5 ppm; 20-25% of the intensity upfield from 5 ppm) can be explained on basis of the differential contributions by the various phospholipid pools mentioned above if one assumes that at 4.7 T (the H_o field strength of WM 200 spectrometer), approximately 90% of the membrane material is oriented and approximately 10% is not oriented (Table 2). Such a percentage of orientation is a reasonable assumption [12].

Table.2. Correlation between spectral intensity distribution in the ^{31}P-NMR spectra of oriented bovine rod outer segments and the morphology of the rod outer segments, based on 90% orientation of all membrane material > 5 ppm = downfield of 5 ppm; < 5 ppm = upfield of 5 ppm.

Rod outer segment substructure	Percentage of total membrane	Chemical shift in ^{31}P-NMR spectrum	Contribution to spectral intensity with 90% orientation	
			>5ppm	<5ppm
	%	%	%	
Flat sides of disks	80-85	$\sigma_{\|\|}$(100 >5ppm)	72-76	
Disk rims	9-12	60-70<5ppm		5- 8
		30-40>5ppm		
Plasma membrane	5- 6	$\sigma_{\perp}$(100 <5ppm)		5
Non-oriented membrane material	10	70-80<5ppm		7- 8
		20-30>5ppm	2- 3	
Total expected spectral intensity			76-83	17-21
Experimentally observed intensity			75-80	20-25

Other important information derives from the resolution of the ^{31}P-NMR spectrum into the major phospholipid classes in the oriented rod outer segment membrane PtdEth, PtdCho and PtdSer. (A) The value obtained for the chemical shift anisotropy of these phospholipids (Table 1) indicates that at 5°C (the lowest temperature measured) each of them has already entered a liquid-crystalline bilayer configuration. This is also consistent with the

value obtained for the spin lattice relaxation time (1.2 s). Apparently the highly unsaturated character of the fatty acids moves the T_m below 5°C. (B) The spectral simulation experiment allows us to conclude that the distribution of PtdEth, PtdCho and PtdSer in the low-field bulk peak, representing the flat part of the disk, is identical to the overal ratio, found for the total rod outer segment [27]. This suggests that none of the three phospholipids has a marked preference for this part of the disk. (C) The phospholipids oriented perpendicular (high field) or parallel (low-field) to the magnetic field exhibit very similar spin lattice relaxation times (T_1). This indicates that either these two distinct lipid pools contain phospholipid material having similar motional dynamics affecting T_1, or that all lipids exchange rapidly between both pools (relative to T_1). Experiments addressing the question of the exchange rate between pools by saturation transfer are in progress. (D) It has been suggested that rhodopsin would have a slight preference for one phospholipid species [34] and this species, therefore, might show restricted motional freedom in the head-group [26]. However, our observations that the T_1 relaxation times of PtdEth, PtdCho and PtdSer do not greatly differ from each other, and that the T_1 equals that expected for a normal liquid-crystalline bilayer, show that the phospholipids detected in our spectra do not show preferential restricted motion. However, if strong immobilization of 15-20 (25-30%) phospholipids by rhodopsin occurred, as suggested by Albert et al. [26], this would strongly reduce their contribution to the resonance intensity in the spectrum. Hence, any such immobilization would also have no marked lipid preference, since the phospholipid composition in our oriented spectra equals that found in complete lipid extracts [25, 27].

Search for non-bilayer contributions

Orientation will influence the resonance position of other lipid phases (hexagonal H_{II}; isotrophic lipidic particle) in a different way from that of the bilayer [18]. Assuming that in hydrated membranes any hexagonal tubes would also from along the plane of the membrane, in analogy to dehydrated photoreceptor membranes [36, 37], orientation will also narrow down the line-shape of the H_{II} phase signal to, and increase its intensity at, its regular peak position (+7 ppm). Isotropic signals will not shift upon orientation and remain at about -2 ppm. The area between -5 ppm and +20 ppm is relatively empty in ^{31}P-NMR spectra of rod outer segments oriented parallel to the magnetic field. This, together with the sharpened resonances from

oriented H_{II} phase structures, allows us to detect under these conditions much smaller amounts of alternative phases than in random oriented membranes. In earlier ^{31}P-NMR studies on random oriented photoreceptor membranes no H_{II} phase was detectable in either non-bleached or bleached samples [23, 25 -27]. Recent electron-microscopic studies on frozen tissue, however, gave some evidence for the occurrence of a small amount of H_{II} stuctures in disk membranes [35]. Dehydration of photoreceptor membranes induces the formation of larger protein free patches of lipids in a hexagonal phase, as demonstrated by electron microscopy and X-ray diffraction studies [36, 37]. However, in our oriented spectra any clear indication for non-bilayer structures could not be detected above the noise level (ca. 1% of total signal intensity) neither in dark-adapted nor in illuminated samples. We conclude that non-bilayer structures are not detectable in isolated intact rod outer segments up to 25°C, a temperature where the isolated lipids already from non-bilayer structures in the presence of millimolar concentrations of divalent cations [27]. Owing to the instability of intact preparations, higher temperatures can not yet be investigated.

Phosphate metabolites

In the area between 0 and -20 ppm any free high-energy phosphates like ATP, ADP, GTP and GDP will appear. We could not find reproducible sharp resonance intensities above the noise level in this region. There was no difference in this respect between pure sucrose or Ficoll/sucrose preparations. We conclude therefore that the total free concentration of these phosphates must be in the submillimolar level, i.e. less than 0.3/rhodopsin. The total level of nucleotides, as determined by chemical analysis, is in the order of 1/rhodopsin. This suggests that the majority of the nucleotides in the rod outer segments are protein-bound.

During incubation, the P_i level in intact preparations slowly rises. This indicates metabolic activity. Therefore, glucose was included in all isolation buffers. Glucose indeed helped the rod outer segments in maintaing their energy charge, which enhanced their structural stability. The presence of significant concentrations of P_i and , possibly, sugar phosphates in isolated rod outer segment preparations has been observed previously [23]. As detected before in less well-defined preparations [23], illumination of the intact preparations also results in a slight shift of the P_i resonance (ca. 0.3 ppm) indicating acidification with about 0.2 pH unit.

Prospects

The ability of isolated rod outer segments to orient spontaneously in the magnetic field of NMR spectrometers opens some interesting lines for further research.

A) Homonucleaer saturation transfer experiments are in progress in order to determine whether rapid, slow or no exchange is possible between the various phospholipid pools. This might, for example, shed some light on the question of whether the disk rims act as a diffusion barrier.

B) Immobilization of oriented rod outer segments in a suitable gel will allow the analysis (spectra, relaxation, phospholipid pools) of a natural membrane oriented with respect to the magnetic field at other angles than 0^{o}. Immobilized rod outer segments, in addition, have much greater structural stability and might be investigated under physiological conditions. Analytical studies of the metabolism of phosphate compounds in intact rod outer segments may then be feasible and could, therefore shed some light on their energy metabolism.

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CHAPTER 7

AN ^{31}P-NMR HOMONUCLEAR SATURATION TRANSFER STUDY ON MAGNETICALLY ORIENTED BOVINE ROD OUTER SEGMENTS : THE ROD OUTER SEGMENT DISC RIM ACTS AS A DIFFUSION BARRIER.

L.C.P.J. Mollevanger, P.A.W. Van Dael and W.J. De Grip

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INTRODUCTION

Recently we demonstrated that carefully isolated bovine rod outer segments retain the capacity to orient in a magnetic field. [1]. The lineshape of ^{31}P-NMR spectra of magnetically oriented rod outer segments was totally different from randomly oriented photoreceptor membranes. The "oriented" spectra were shown to represent three phospholipid pools. A major low-field peak (75-80% of the total intensity) near 30 ppm was attributed to the flat part of the disk membranes, and a minor high field peak (10-15%) near -14 ppm to phospholipids of the plasma membrane and disk rim phospholipids, which are oriented perpendicular to the flat part of the disk. Disk rim phospholipids would further contribute intensity to the middle part of the spectrum. These major pools were further partially resolved with respect to phospholipid headgroup class [1].

This unique situation (three lipid pools in different chemical shift ranges + partial resolution of headgroup classes) affords several interesting lines of further investigation [1]. In this contribution we elaborate the feature that diffusional exchange between phospholipid pools exhibiting different chemical shifts can be studied by ^{31}P-NMR spectroscopy using the homonuclear saturation technique.

Homonuclear saturation transfer involves the transfer of saturation from one chemical environment to another characterized by a different chemical shift. Experimentally this means that spins in a particular environment (i.e. at a particular chemical shift) are saturated by a selective radiofrequency pulse. Subsequently the transfer of saturation to the other chemical environment (different chemical shift) is detected as a decrease in peak intensity at that position. This technique can be used to study dynamic processes with exchange times between a milli-second and a second. In biological systems it can e.g. be applied to study turnover-rates of water soluble phosphate metabolites, which are involved in the energy supply [2]. Also it allows the suppression of the very broad (approx. 50 ppm) signal from the membrane phospholipids which distorts the narrow peaks of water soluble metabolites in "in vivo" ^{31}P-NMR spectroscopy of cells or whole organs [3]. Furthermore, it can be used to study lateral diffusion of phospholipids in artificial and biological membranes [4,5]. In this study we apply homonuclear saturation transfer in ^{31}P-NMR spectroscopy of the photoreceptor membrane to study phospholipid exchange between the three different phospholipid pools,

which can be distinguished in magnetically oriented rod outer segments [1].

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade. Egg phosphatidyl choline (grade 1) was purchased from Avanti Lipids Inc. and NMR tubes (ø = 10mm) from Wilmad Inc. (Buena, N.J., USA). The "isolation buffer" contained: 20 mM Mops, 130 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 10 mM Glucose, and was adjusted to pH = 7.2 with 1 M NaOH. The "NMR-buffer" had the same composition as the isolation buffer, but contained in addition 20% v/v ^2H_2O, for the deuterium lock of the NMR spectrometer, 0.02 % NaN_3 as a bacteriostatic agent and 5% w/v sucrose, in order to increase the density of the medium and retard sedimentation of the rod outer segments during NMR measurements.

Isolation of bovine rod outer segments

Efficient orientation of rod outer segments depends heavily on an intact regular disk stacking. Therefore it is essential to isolate the rod outer segments as intact as possible. Morphologically intact bovine rod outer segments were isolated from freshly excised retinas by means of sucrose density gradient centrifugation using the protocol we previously developed [1].

Sample preparation for NMR

Low speed rod outer segment membrane pellets were carefully resuspended in NMR buffer to a final concentration of 800 nmol rhodopsin/ml using a pipettor with a polyethylene tip. By means of a disposable pipette 1.4 ml of this suspension was transferred into a 10 mm NMR tube.

Preparation of egg phosphatidylcholine suspension

A solution of egg phosphatidyl-choline as commercially supplied (50 mg/ml in chloroform/methanol) was transferred to a small glass round bottom flask and the solvent was evaporated under vacuum. The remaining lipid film was dried for several hours under high vacuum to remove all solvent residues. Subsequently 2 ml of NMR buffer was added to the dried lipid film together with some glassbeads. By gentle aggitation a liposome suspension was formed, which contains large multilamellar liposomes. One quarter of this suspension was transferred to a small glass tube and was sonicated for two minutes under ice-cooling using a Branson B12 sonifier with microtip at medium power level.

This resulted in a clear suspension of very small unilamellar vesicles (diameter < 50 nm).

NMR Measurements

^{31}P-NMR measurements were performed on a Bruker WM 200 spectrometer operating at 81 MHz, and equipped with a dual ^{13}C-^{31}P 10 mm probe. Observation-pulses of 15 µsec (60°) were used, with a 16 kHz sweepwidth , and with an interpulse delay of 1 sec. Two-level broadband ^{1}H decoupling, 3 W during data aquisition (0.12 sec) and 0.3 W during the relaxation delay, was applied. To improve the signal to noise ratio, an exponential filter of 50 Hz was applied to the accumulated free induction decays before Fourier transformation. All shifts are relative to external H_3PO_4.

For the homonuclear saturation transfer experiments a second synthesizer (PTS 160) was used to generate a selective long saturation pulse. The synthesizer was connected to a RF amplifier of which the output could be adjusted with increments of 1 dB. The output was under computer control and fed through a directional coupler directly into the ^{31}P probe. A saturation pulse of one second duration was given at a selected frequency preceding each observation pulse.

The saturation transfer spectra and control spectra were recorded by alternatingly accumulating 100 FID's with and 100 without saturation. Subsequently similar blocks of 100 FID'S were added separately. This assures that both spectra are recorded under identical experimental conditions. For each spectrum in total about 4000 transients were accumulated.

RESULTS AND DISCUSSION

Performance test of the saturation unit

In order to test the saturation unit and to optimize the conditions for saturation transfer, a model phospholipid system was used as a test case. Hereto a multilamellar liposomal dispersion of egg phosphatidylcholine was mixed with a dispersion of small vesicles of the same lipid prepared by sonication. ^{31}P-NMR spectra of the large liposomes show the familiar broad bilayer lineshape ([7], cf. Fig. 1 insert), while the small vesicles thanks to their small rotational correlation time and small diameter allow isotropic averaging of the phosphate-chemical shift anisotropy and yield a narrow line at 0 ppm ([7], cf. Fig. 1 insert). In a mixture of these two systems , showing a narrow line superimposed on the broad bilayer resonance signal (Fig. 1 A),

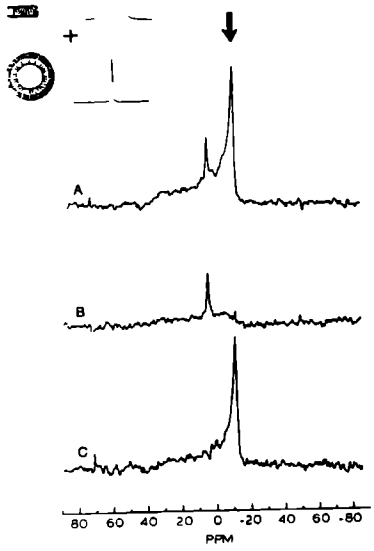

Figure 1 : Egg-phosphatidylcholine model system demonstrating the principles of homonuclear saturation transfer. A) The ^{31}P-NMR spectrum of a mixture of large liposomes and small unilamellar vesicles (see insert) suspended in 20 mM Mops buffer, pH 7.2. Total lipid content is 40 µmol/ml. The arrow indicates the saturation frequency employed in B. B) The ^{31}P-NMR spectrum of the same sample obtained after homonuclear saturation (1 sec, 0.3 Watt, preceding the observation pulse). Both spectra are an accumulation of 600 FID's, and a 15 µsec observation pulse was used with an 1 sec interpulse delay. C) Difference spectrum of A - B, which represents the components saturated in B.

no exchange occurs between the two phospholipid populations [3]. Hence, if the liposomal spin system is saturated at a frequency sufficiently different from the isotropic position, only the lyposomal resonance should be affected without significant change in the isotropic line. This can be explained as follows:

In a large fluid vesicle the phospholipids continuously change chemical shift due to lateral diffusion. In this way, phospholipids can transfer saturation from the narrow spectral region in which the spin system is saturated, to every other position accessible to exchange. With a lateral diffusion constant of ca. 10^{-7} cm^2/sec, typical for phospholipids in a fluid bilayer [8], it takes a phospholipid several tenths of a second to "cover" a liposome of 1 µm diameter. Once outside the saturation bandwidth, the irradiated phospholipid spins will return to the equilibrium distribution with a relaxation time of about 1.2 sec [1]. However, for sufficiently long saturation pulses the influx of saturated phospholipids is so large that the thermal equilibrium distribution can no longer be attained. As a result a steady state situation is reached, in which the signal from spins at all chemical shift positions, that exchange with spins resonating at the saturation frequency, is reduced in intensity. This would mean for the

model-system of figure 1, that under optimal conditions selective saturation in the bilayer peak would lead to the elimination of the liposomal signal, but would leave the vesicle-peak unaffected. Figure 1B demonstrates that this situation indeed can be achieved with the present set-up by irradiation at the high-field peak position of the bilayer signal with saturation pulses of 0.3 Watt intensity and at least 1 sec duration. Under these conditions no significant decrease in the narrow line of the small vesicles is detected (Fig. 1C). Lower pulse intensities or shorter pulse lengths resulted in only partial elimination of the bilayer signal.

Irradiation at the low-field shoulder does not lead to complete elimination of the bilayer-signal. This is due to the fact that the spin density contributing to the signal at this position is relatively small. As a result the output of saturated phospholipids is too low to achieve complete saturation of the remainder of the phospholipid pool since the longitudinal relaxation (1.2 sec) is sufficiently rapid to partially re-equilibrate the spin system. Longer saturation pulses are not of any help here, since it was observed experimentally that the steady state, determined by saturation-pulse length, relaxation time and lateral diffusion rate, is reached in 0.5 to 1 sec.

Photoreceptor membranes.

Since the photoreceptor membranes differ from egg phosphatidylcholine liposomes in size-distribution (large, uniform, monolameller vesicles, ø ca 1 um) and fluidity (high content of polyunsaturated lipids, [9,10]) , and additionally have a high protein content, the saturation transfer conditions developed for the model system were first tested on dispersions of isolated photoreceptor membranes before turning to intact outer segments. Hereto a somewhat similar approach as for the egg-PC system was used. Hypotonic lysis of rod outer segments yields a dispersion of photoreceptor membrane vesicles mixed with soluble components, including inorganic phosphate and organic phosphate mono-esters, which do not exchange rapidly with phospholipid phosphate (1,11). These soluble components generate two sharp ^{31}P resonance lines near 0 ppm superimposed on the broad bilayer signal of the vesicles (Fig. 2A). Saturation experiments carried out with this system at the conditions described above yielded results very similar to the egg-PC system. Irradiation at the high field peak position (0.3 Watt intensity; 1 sec pulse length) eliminated the bilayer signal (Fig. 2B) without a measurable effect on

the sharp metabolite resonances (Fig. 2C). Irradiation at the low-field shoulder did not entirely eliminate the bilayer signal (Fig. 2D). However, maximally only 20% of the total intensity remains, mainly at high field (cf. Fig. 2A,D,E.). Hence, even upon irradiation at frequencies corresponding with low spin density, over 80% of the total spin population can be saturated. As expected, this figure could not be further raised by longer saturation pulses.

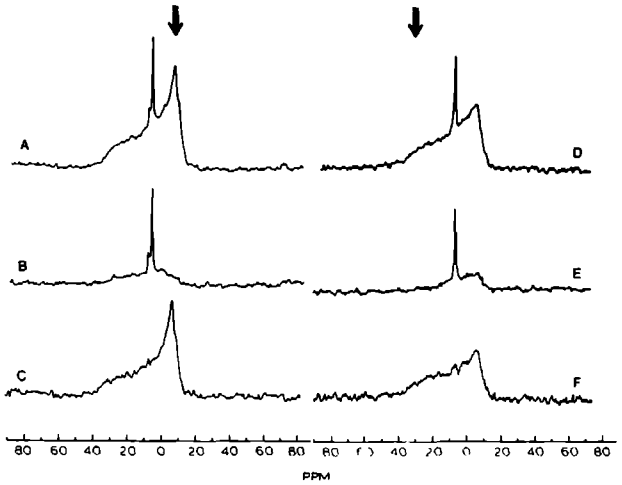

Figure 2: Homonuclear saturation of spins in the photoreceptor membrane bilayer by irradiation at the high-field (A to C) and low-field (D to F) side of the spectrum. A) and D) show ^{31}P-NMR spectra of hypotonically lysed bovine rod outer segments (monolamellar vesicles with a diameter of approximately 1 µm) without saturation. The isotropic peaks originate from small phosphate metabolites. B) and E) show ^{31}P-NMR spectra of the same samples obtained following an 1 sec (0.3 Watt) saturation pulse at the spectral position indicated by the arrows in A and D, respectively. C) and F) show difference spectra representing the spectrum of the saturated components components in B and E respectively: C represents A - B and F represents D - E.

Oriented rod outer segments

General aspects:

Intact rod outer segments orient with their long axis parallel to the magnetic field in field strengths exceeding 1 Tesla (frog, [12]) or 4 Tesla (cattle, [1]). Figure 3 shows the typical ^{31}P-NMR spectrum of intact, magnetically oriented, bovine rod outer segments together with morphological

assignments [1]. The major low-field peak represents the flat parts of the disk membranes, in which the phospholipids are oriented parallel to the magnetic field. The minor high-field peak mainly represents the phospholipids in the plasma membrane, which are oriented perpendicular to the magnetic field. The phospholipids in the disk rim structure occupy a curved structure and will generate a broad bilayer-like signal, which contributes to the middle part of the spectrum.

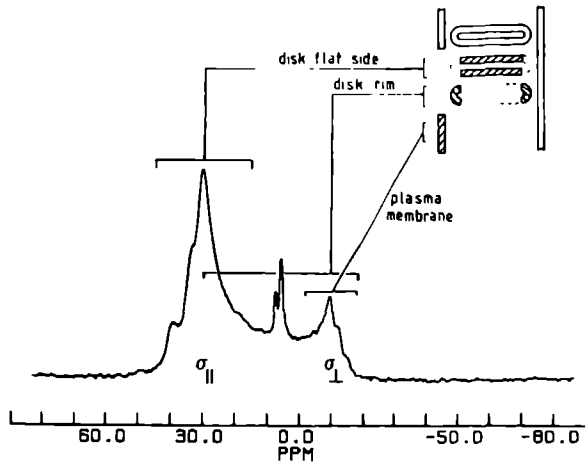

Figure 3: Correlation between the peak distribution in ^{31}P-NMR spectra of magnetically oriented bovine rod outer segments and morphological substructures [1].

The disk rim has a relatively high curvature for a biological membrane and stabilization by specific rim-proteins [13,14], or skeletal elements [15,16] has been suggested. This could indicate a rather special micro environment for the lipid matrix in the disk rim. Skeletal element probably also connect disk and plasma membrane [16], but so far there is no information as to whether these phospholipid pools do actually exchange. The oriented intact rod outer segment offers the unique situation that these three pools (disk flat, disk rim and plasma membrane) can be isolated and studied by ^{31}P-NMR spectroscopy in the intact organelle. We have employed the saturation transfer technique described above to investigate whether at 5° C free exchange of phospholipids between these pools can take place. Higher temperatures could not yet be examined due to the structural fragility of the intact rod outer segment [1]. Although the result obtained at 5° C cannot be directly extrapolated to physiological conditions, they are free of metabolic interference and will sketch a globally correct picture, since in fluid

bilayers the lateral diffusion coefficient is not very strongly temperature-dependent [8].

Irradiation at the disc flat side:

Irradiation at the major, low-field peak, under the standard conditions described above, completely eliminates this peak (Fig. 4B). This resonance consists of three overlapping subpeaks representing the phospholipid headgroup classes phosphatidyl ethanolamine, phosphatidylcholine and phosphatidylserine [1]. We were therefore curious whether one headgroup class could be selectively saturated. However, even when we carefully aimed at the low-field subpeak, which represents phosphatidylserine, considerable elimination of the phosphatidylcholine and phosphatidylethanolamine subpeaks was detected (not shown). This might be caused by the still considerable overlap between these peaks.

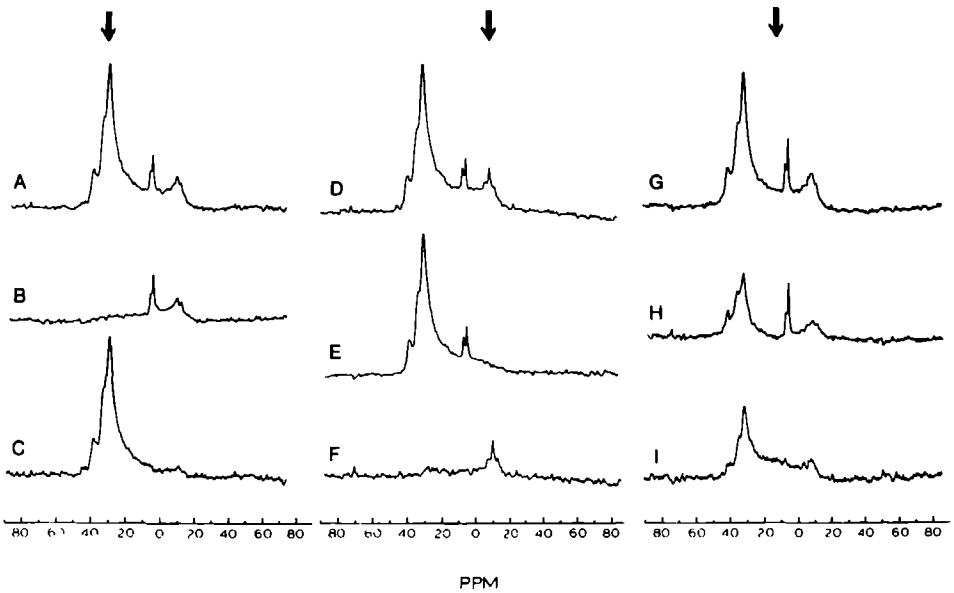

Figure 4 : ^{31}P-NMR spectra of intact oriented bovine rod outer segments with (B, E, H) and without (A, D, G) homonuclear saturation at the spectral position indicated by the arrows in A, D and G respectively. Spectra C), F) and I) show difference spectra (A - B, D - E and G - H) respectively and represent the spectrum of the respectively saturated components. Each spectrum is an accumulation of 4000 FID's.

Under the employed saturation conditions, irradiation at a site corresponding with high spin density should completely saturate all phospholipid pools which can rapidly exchange with this site (Fig. 1B, 2B). Indeed the entire low-field peak can be eliminated in this way (Fig. 4B). However neither the middle part of the spectrum nor the high-field peak are strongly affected (Fig. 4C). This indicates that the preparations contain very little randomly oriented material (< 3%) since this would yield a normal bilayer spectrum [1], which would be nearly completely eliminated (Fig. 2E). The result also suggests that not only exchange between disk flat and plasma membrane is very restricted if not impossible (which is not too surprising) but also that no rapid exchange between disk flat and disk rim occurs. This remarkable finding should be substantiated by selective saturation of these other pools.

Irradiation at the plasma membrane:

Irradiation at the minor high-field peak position also leads to entire elimination of this peak (Fig. 4E). Again the middle part of the spectrum is barely affected and the reduction in intensity of the low-field peak is less than 5%. It is likely in view of the previous experiments (Fig. 4B) that the disk and plasma membrane pools do not exchange. If the disk rim pool, which contributes up to about 50% of the intensity of the high-field peak [1], would be in rapid exchange with the disk flat side, one would expect that irradiation at the position given in Fig. 4E would strongly affect the middle part of the spectrum and reduce the intensity of the low-field peak by at least 30 to 50% (cf. Fig. 1C, 2E). Both effects are not observed and again indicate restricted diffusion between disk rim and disk flat side.

Irradiation at the disk rim:

Irradiation at the middle part of the spectrum is less straightforward in view of the fairly broad base of the low-field peak (cf. Fig. 4B), so that direct irradiation of this peak should be taken into account. Nevertheless the general features confirm the results described so far (Fig. 4G,H,I). Irradiation just down-field from the sharp resonances of the soluble phosphates eliminates the middle part of the spectrum. Furthermore, both the low-field peak and the high-field peak are reduced in size by about 50%, while the soluble resonances are not affected at all.

The reduction in the high field peak is accordance with our earlier estimate that the disk rim contributes at least 50% of the intensity in this part of the spectrum [1]. The shape of the difference spectrum (Fig. 4I),

which represents the saturated components, is unusual in that its high field part does not show the regular bilayer shape. In our opinion this agrees with the earlier results, indicating that there is restricted phospholipid diffusion within the disk rim. The only partial reduction of the low-field peak lends further support to this conclusion. In the case of free exchange between the "saturated" part of the disk rim and the disk flat, the low-field peak should be saturated by at least 80% (Fig. 2B), which is not observed. It is presently not clear whether the still 50% reduction in size of this peak reflects less restrictions in exchange between intermediate parts of the disk rim and the disk flat as compared to the extreme part of the disk rim and the disk flat (cf. Fig. 4F vs. 4I) or is due to the fact that the extreme base of the low-field peak already is saturated at this position.

CONCLUSIONS

The homonuclear saturation transfer technique in combination with orientation of the rod outer segment gives access to information which is difficult to obtain otherwise. The developed saturation unit allows complete saturation even of a very broad lineshape like the liposomal bilayer signal by irradiation at a high field peak frequency with 0.3 Watt intensity and pulse-lengths of one second. Under these conditions no non-selective saturation effects could be detected. Irradiation at frequencies corresponding to low spin density still leads to a reduction of the intensity of the entire bilayer signal by at least 80%. ^{31}P-NMR spectroscopy of magnetically oriented bovine rod outer segments resolves the three main phospholipid pools: disk flat, disk rim and plasma membrane [1]. This situation permits the use of homonuclear saturation transfer to investigate whether free exchange of lipid molecules between these pools occurs. Irradiation at a spectral position of either the disk flat or plasma membrane pool signal gives rise to saturation of the spins in the respective pools, but has little effect on those in the other pools. Irradiation at the spectral position of the disk rim signal does not completely saturate the spins in this pool and has variable effects on the spins in the other two pools. We conclude that (Fig. 5) :

1) Rapid lateral diffusion of phospholipids is allowed within the flat part of the disk membrane and within the plasma membrane.

2) The physical separation of plasma membrane and disk membrane indeed prevents rapid exchange, if any at all, of phospholipids by diffusion.

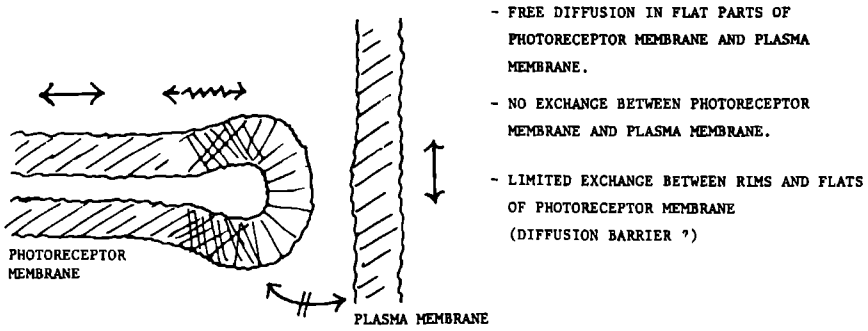

Figure 5 : Schematic view of the possible diffusional exchange routes for rod outer segment phospholipids. <---> means unrestricted exchange by diffusion on the NMR timescale, <-┼-> means that exchange by diffusion is not possible or very slow, <~~~> means restricted exchange by diffusion barrier.

3) The disk rim acts as a diffusion barrier for phospholipids, which slows down lateral diffusion between flat and rim parts of the disk. The question, whether this barrier is formed by special intrinsic rim proteins, required to stabilize the rim structure, or by interaction with extrinsic membrane bound proteins like cytoskeletal elements or specially positioned signal mediating proteins (like the cGMP phosphodiesterase) affords an interesting line for further research.

4) Qualitatively, we can state that lateral diffusion of phospholipids in the disk rim is slowed down as compared to the flat part of the disk. We therefore predict that the rim presents an absolute diffusion-barrier for much larger structures like the intrinsic membrane protein rhodopsin. This would mean that the freedom of movement of rhodopsin is restricted to one side of the disk membrane only.

5) Earlier observations [17,18] indicate that the disk rim indeed has a special structure. It would be relevant to know whether this special structure is retained upon hypotonic lysis of the disk which inflates the flat "double membrane apposition" into the familiar spherical vesicle shape [19]. This aspect cannot very well be studied by saturation transfer because these vesicles are randomly dispersed. If the special rim properties are lost upon inflation, the diffusion barrier is probably eliminated and free diffusion of

all membrane components over the entire disk surface is allowed. This could explain why it is very difficult to deflate such inflated disks back into the regular flat disk structure. Further this phenomenon might be relevant to degenerative retina conditions which are often accompanied by formation of vesicular disk membranes [20,21,22].

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HIGH RESOLUTION SOLID STATE ^{13}C-NMR STUDY OF CARBONS C-5 AND C-12 OF THE CHROMOPHORE OF BOVINE RHODOPSIN : EVIDENCE FOR A 6-S-CIS CONFORMATION WITH NEGATIVE CHARGE PERTURBATION NEAR C-12.

Leo C.P.J. Mollevanger, Arno P.M. Kentgens,
Johannes A. Pardoen, Jacques M.L. Courtin,
Wibren S. Veeman, Johan Lugtenburg and Willem J. de Grip

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INTRODUCTION

Rhodopsin is the photosensory pigment of the photoreceptor membrane in the rod cell of the vertebrate retina. It is a amphipathic intrinsic membrane protein comprised of the apoprotein opsin and a chromophoric group. Opsin is a single chain polypeptide of 348 amino acids (40 kDa M_r.) [1,2,3]. Rhodopsin owes its visible absorption band peaking at 500 nm to the covalently bound chromophoric group: 11-cis retinal (Fig. 1B) [4]. The primary event in vision is photon capture by rhodopsin which induces isomerisation of 11-cis retinal into the all trans configuration. This provokes conformational changes in the interior of the protein which propagate to the outside and create topological changes on the surface of rhodopsin.The latter are detected by elements of a signal transduction/amplification cascade, which finally leads to the closing of sodium channels in the plasma membrane of the rod cell within several hundred msec after photon capture. For a review see [5,6].

When 11-cis-retinal binds to opsin under formation of rhodopsin a large red-shift occurs in its absorption maximum (378 nm to 500 nm). In rhodopsin the chromophore is bound via a Schiff base bond to the ε-amino group of Lysine 296 [1,2,7]. Resonance Raman spectra indicate that this bond is protonated in native rhodopsin [8]. Model protonated Schiff bases of retinal absorb around 440 nm and apparently formation of a protonated retinal Schiff base per se cannot explain the absorption maximum of rhodopsin (500 nm) [9,10]. The difference in wavenumber between rhodopsin and a model protonated Schiff base is called "opsin shift". For rhodopsin it amounts to : $10^7 * (1/440 - 1/500) = 2730\ cm^{-1}$. This so called "opsin shift" is not unique for rhodopsin but is observed in all photosensory pigments based on retinal. Its magnitude however depends upon the actual pigment and nature developed a mechanism to construct photopigments which cover the spectral range from 430 to 560 nm [11,12].

The mechanisms by which a protein moiety (opsin) might significantly influence the absorption maximum of a protonated Schiff base of retinal can be divided into two catagories: 1) Conformational changes forced upon the retinal moiety (e.g. twisting around intramolecular bonds). For instance Honig et al. [13] estimated that twisting around the 6-7 single bond in a protonated retinal Schiff base can produce a red shift up to 50 nm . 2) Electrostatic interaction with charged protein residues [9,10,13,14]. This second mechanism was first proposed in 1958 by Kropf and Hubbard [14]. It was pointed out by Honig et al. [13] that this would require at least two point charges in order

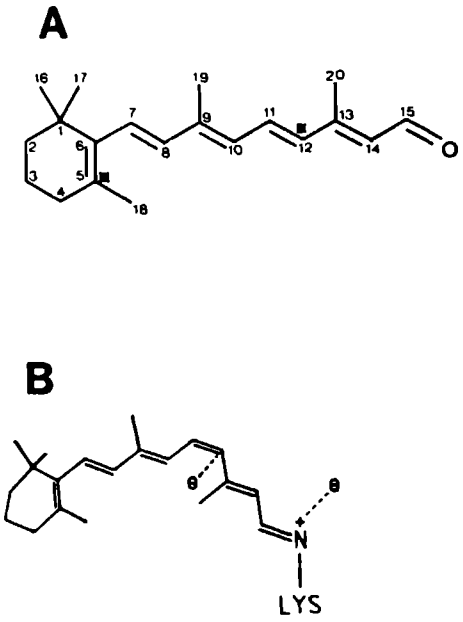

Figure 1 : (A) Structure of all trans retinal showing the numbering of atoms and the positions of the labels in this study. (B) The external point charge model as an explanation for the opsin shift of the protonated retinylidene in bovine rhodopsin as proposed by Honig , Nakanishi and coworkers [9,10].

to explain the observed opsin shifts. One negative charge, as a counterion, near the protonated Schiff base and a second negative charge or strong dipole along the retinylidene polyene chain would satisfy this criterium . Based on the spectroscopic properties of several dehydroretinal rhodopsin analogs [15] Honig, Nakanishi and coworkers [16] developed a model in which the second negative charge is placed near carbon-12 of the polyene chain (Fig. 1B [10]).

Harbison et al. [17,18] already demonstrated for bacteriorhodopsin that ^{13}C high resolution solid state (MAS) NMR spectroscopy is a very powerful, non invasive technique to probe the structure of the chromophore (configuration, conformation, charge distribution and interaction with charges of the peptide chain) in the active site of photosensory pigments . In this paper we will demonstrate the first application of this technique to bovine rhodopsin. Hereto we used rhodopsin which was ^{13}C enriched in its chromophore by incorporation of 11-cis retinal selectively labeled at either carbon C-5 or C-12 of the polyene chain. Careful analysis of our data leads us to the conclusion that the C-6 - C-7 single bond of retinal in bovine rhodopsin is in the s-cis conformation. Our data also suggest the presence of a negative charge in the proximity of carbon atom C-12.

^{13}C labeled retinals.

11-cis retinals were selectively enriched over 90% with ^{13}C either at the C-5 or the C-12 position as described by Pardoen et al. and Courtin et al. [19,20].

Preparation of rhodopsin containing ^{13}C labeled retinal.

Most procedures have been described before in detail [21]. In brief: Bovine rod outer segment membranes were isolated in the illuminated form (opsin), using a continuous sucrose density gradient procedure ,from fresh retinas homogenized in the light in the presence of NADPH. The resulting rod outer segment opsin membranes can be stored as a pellet for several weeks at -70 ^{o}C.

For the formation of labeled pigments, an equimolar amount of the selectively ^{13}C labeled 11-cis retinal was added as a concentrated ethanol solution to rod outer segment opsin membranes resuspended in aqueous buffer (20 mM Pipes, pH= 6.5). The formation of pigment was followed spectrofotometrically at 520 nm. In order to remove any aspecifically bound retinal as well as the highly unsaturated lipids, which both interfere with the ^{13}C-NMR analysis, labeled rhodopsin was purified by affinity chromatography over Con A-Sepharose (Pharmacia, Uppsala, Sweden) [22]. The purified labeled rhodopsin was reconstituted in a lipid/rhodopsin ratio of 30-40 : 1 with diphytanoyl-PtdCho (Avanti Polar Lipids Inc. Birmingham AL. U.S.A.) by the detergent dilution method [23].

The type spinners we used (see below) became highly unstable when filled with aqueous membrane suspensions. Therefore the membrane preparations were lyophilized before they were transferred to the spinner. Under the proper conditions lyophilization does not affect the spectral properties of membrane bound rhodopsin [24]. Rod outer segment membranes that were rehydrated after lyophilization show a normal photolytic cascade. Furthermore Harbison et al. [17] did not detect significant differences between the high resolution CP MAS ^{13}C NMR spectra taken of fully hydrated and lyophilized bacteriorhodopsin containing purple membranes.

Recording of solid state CP-MAS ^{13}C-NMR spectra.

High resolution solid state CP-MAS ^{13}C-NMR spectra were obtained on a Bruker CXP-300 NMR spectrometer working at 75 MHz for ^{13}C nuclei. The rotors were of the Andrew-Beams design and were constructed from the industrial

ceramic boronitride (BN). These do not show ^{13}C signals which is a disturbing aspect of the more routinely used Delrin roters. Cross polarisation from ^{1}H to ^{13}C spin systems was achieved with rotating fields of 15 and 60 Gauss respectively and a mixing time of 2 msec. A 2 second relaxation delay between pulses was used. Spectra were recorded at different spinning speeds between 2.1 and 2.5 kHz. The ^{1}H decoupling field was equivalent to 60 kHz. To prevent baseline distortions phase cycling was used combined with spin temperature alternation (cyclops) . All chemical shifts are relative to external tetramethylsilane (Me_4Si).

Calculation of chemical shift tensor elements

Values for the chemical shift tensor elements were calculated from the intensity distribution between the centre band and the rotational sidebands identified in the MAS-NMR spectra, according to the graphical approach developed by Herzfeld and Berger [25]. Calculations were performed on spectra obtained at two different spinning speeds (2.1 and 2.5 kHz). Hereto the centre band and first and second order side bands in the spectra were fitted with a gaussian lineshape and their area was determined graphically. In principle, the two first order sidebands are already sufficiant to calculate the tensor elelments, but additional sidebands were used to check or refine the obtained values. Thus, the final accuracy in the values calculated for the ^{13}C tensor elements was substantially better then the accuracy with which the intensity of each individual band could be obtained. Analysis of the calculations and comparison of values obtained at different spinning speeds, indicated an upper level of $\pm$ 6 ppm for the error in the values of the tensor elements. It also showed that the values for σ_{22} and σ_{33} were less susceptible to variations in the intensity measurements than the value for σ_{11}.

In spite the fact that we concentrate on only one particular C atom out of a complex of 3500 C atoms the signal to noise ratio of the centre band and first rotational sidebands is reasonably good. The signal to noise ratio of our spectra is comparable to similar spectra published for bacteriorhodopsin, see [17] and the latter authers arrive at a comparable accuracy in the values of the tensor elements.

RESULTS

Figure 2 shows typical MAS ^{13}C NMR spectra of dark adapted lyophilized rod outer segment membranes under various conditions. A conventional ^{13}C-MAS

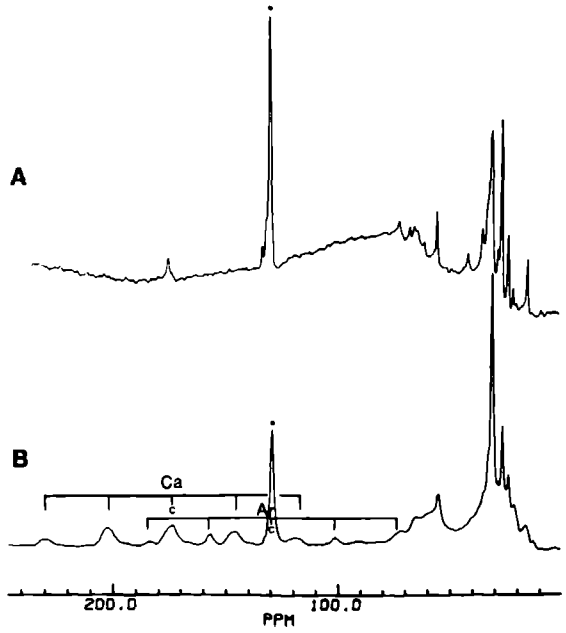

Figure 2 : ^{13}C-MAS NMR spectrum of lyophilized rod outer segment membranes measured with (A) 90 ^{o} pulses and (B) ^{1}H-^{13}C cross polarization. Spectra were taken using a sample spinning speed of 2.1 kHz and each spectrum is an accumulation of approximately 30000 FID's. For spectrometer settings see the Method section. Samples contained ca. 450 nmol of rhodopsin. (Ar) = aromatic centerbands (c) and rotational sidebands (Ca) = carbonyl centerbands (c) and rotational sidebands

spectrum, recorded with 90^{o} pulses, is given in figure 2A. It shows an abundance of sharp resonances which all derive from the mobile lipid acyl chains, since they disappear following lipid extraction (not shown). The rather immobile protein resonances of carbonyl and aromatic residues probably relax too slow and do not show up in these spectra. The intensity of less mobile groups can however selectively be enhanced by ^{1}H-^{13}C cross-polarization. With this approach (Fig. 2B) characteristic protein features like the resonances of the carbonyls at 172 ppm and of the aromatic residues at 128 ppm now appear clearly, together with strong rotational sidebands. Both give relatively broad resonances because of chemical heterogeneity of the carbonyl and aromatic groups in the protein and in the

case of the carbonyls also as a consequence of dipolar coupling to ^{14}N in the peptide bond [26]. Very apparent is the intense resonance peak at 128.5 ppm. This resonance has been assigned to the double bond CH=CH carbon atoms in the fatty acyl chains of the phospholipids [27] in view of the very high content of polyunsaturated fatty acids in rod outer segment membranes [28,29] and indeed disappears following lipid removal (not shown). The resonances from the ethylenic ^{13}C labeled positions in retinal are also expected to occur between 125 and 145 ppm [17,18] and a relatively intense resonance at 128.5 ppm therefore will seriously hinder the analysis of the resonances in spectra of rhodopsin in which labeled retinal has been incorporated.

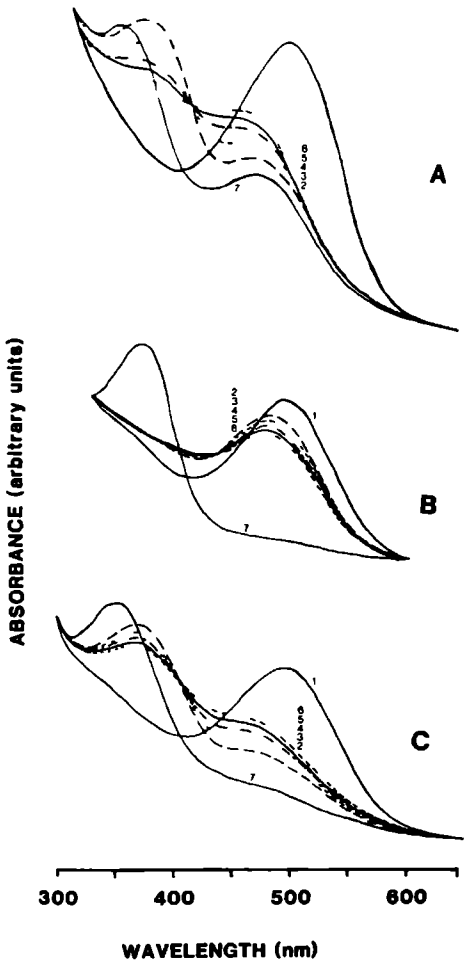

Figure 3 : Photolysis of rhodopsin in diphytanoyl-PtdCho compared to native membranes. Absorbance spectra of a suspension of photoreceptor membrane (A) or rhodopsin reconstituted in either distearoyl-PtdCho (B) or diphytanoyl-PtdCho (C), all suspended in 20 mM Pipes-buffer, pH=6.5, 20 ^{o}C, (spectrum 1). After flash illumination (spectrum 2), subsequent spectra (3-7) were recorded every 3 minutes. (A) and (C) show the normal sequence: Metarhodopsin II (380 nm) is generated immediately upon illumination and slowly decays under formation of metarhodopsin III (455 nm). For further details see [23].

Hence it was necessary to remove the unsaturated lipids. However, lipid-free rhodopsin is a less suitable substrate for this kind of investigation, as it is not very stable and does not very well survive lyophilization [24]. The best solution therefore is to exchange the unsaturated lipids for saturated ones via a purification and reconstitution step . A complicating factor occurs here because reconstitution in fully saturated straight chain phospholipids affects the photochemical behaviour of rhodopsin [30]. Instead we therefore have selected the fully saturated branched-chain phospholipid diphytanoyl-PtdCho (acyl chain: 3,7,11,15-tetramethylhexadecanoic acid), in which rhodopsin shows fairly normal photolysis (Fig. 3). The presence of the four bulky methyl groups probably prevents the close packing adopted by phospholipids with straight saturated acyl chains, and creates a more fluid environment comparable to lipids with unsaturated acyl chains [31].

Figure 4 compares the CP-MAS ^{13}C NMR spectra of (a) Pure diphytanoyl-PtdCho, (b) Unlabeled rhodopsin reconstituted in diphytanoyl-PtdCho, and rhodopsin labeled with (c) ^{13}C-5 or (d) ^{13}C-12 11-cis retinal which, following purification, was reconstituted in diphytanoyl-PtdCho. Only the low field side of the spectra is shown. The intense peak at 128.5 ppm present in the spectra of rod outer segment membranes (Fig. 2) has now been very strongly reduced (asterix). In the spectra of the labeled rhodopsins new resonances are apparent in the region between 100 and 160 ppm (arrows), which derive from the single ^{13}C-labeled retinal . This is very evident in the difference spectra shown in figure 5 A and B obtained by subtracting the spectrum of non labeled rhodopsin (Fig. 4B) from the spectra of the labeled rhodopsins (Fig. 4C, 4D). The centre and side bands of the new resonances were identified by analysis of spectra recorded at different spinning speeds. The isotropic chemical shifts of the carbons C-5 and C-12 are located at respectively 130.5 ppm and 133.5 ppm ($\pm$0.3 ppm). Several spinning side bands can be observed additional to the centre band, indicating a large ^{13}C chemical shift anisotropy for both nuclei. Bleaching of the samples (300 Watt tungsten lamp through a 530 nm cut-off filter) resulted in a mixed population of photoproducts. In the spectra this caused a large decrease in the sharp resonances assigned to C-5 and C-12, and a complete loss of the spinning side bands (Fig. 5C). The decrease of resonance intensity at σ_1 is probably due to line broadening as a result of chemical heterogeniety provoked by the bleaching.

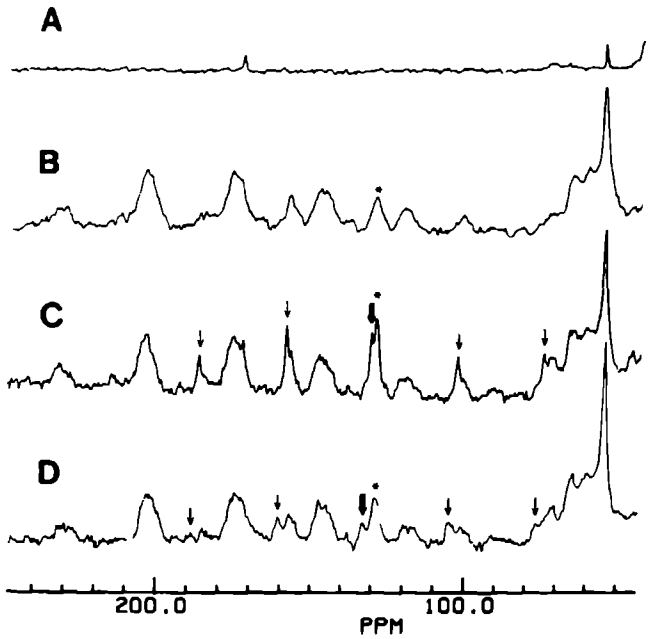

Figure 4 : ^{13}C MAS-NMR spectra of lyophilized samples of ; (A) diphytanoyl-PtdCho. (B) Bovine rhodopsin in diphytanoyl-PtdCho. (C) ^{13}C-5 Retinylidene bovine rhodopsin reconstituted in diphytanoyl -PtdCho. (D) ^{13}C-12 Retinylidene bovine rhodopsin in diphytanoyl -PtdCho. Spectrometer settings as in the Method section. Each spectrum is an accumulation of 30000 FID's. Samples contained 450 nmol of labeled rhodopsin. (*) = Resonance arising from residual unsaturated fatty acid. The amount slightly varies between preparations. (↓)= Centerband of the labeled retinyl resonance. (↓)= Rotational side bands of the labeled retinyl resonance.

Useful information on the molecular environment of a nucleus can be obtained by resolving its chemical shift tensor elements, and comparing this to model systems [18]. Recently Herzfeld and Berger [25] developed a graphical approach to derive the values for the principal chemical shielding tensor elements from the intensity distribution between the centre band and the rotational sidebands in NMR spectra recorded at the magic angle. We used this approach to calculate the tensor elements σ_{11}, σ_{22} and σ_{33} of the ^{13}C-5 and ^{13}C-12 nuclei of retinal in rhodopsin, from spectra recorded at two spinning speeds (2.1 and 2.5 kHz). This yields values of 33 ppm, 143 ppm and 215 ppm for carbon C-5 and 41 ppm, 149 ppm and 209 ppm for carbon C-12

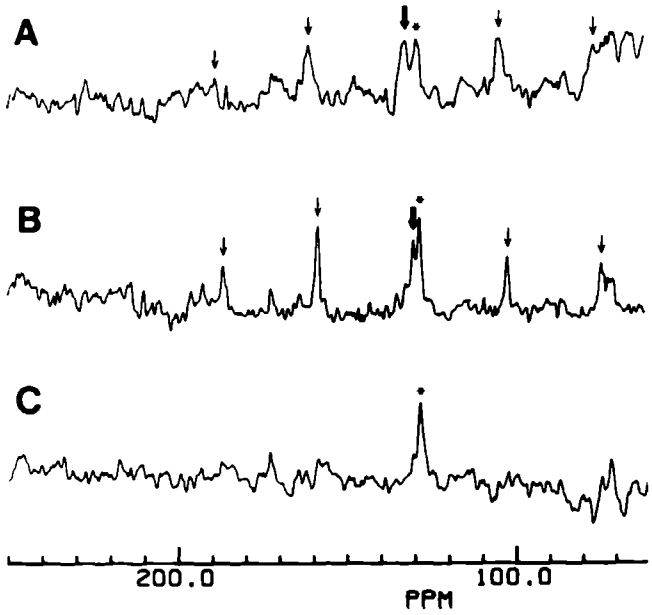

Figure 5 : Identification of the ^{13}C labeled retinyl resonances in bovine rhodopsin, by subtraction of the spectra in figure 4. (A) Resonances of carbon ^{13}C-12. (fig.4 D minus fig.4 B). (B) Resonances of carbon ^{13}C-5. (fig.4 C minus fig.4 B). (C) Like (B), but now after illumination of the sample.

respectively. The error in these values is in the order of 6 ppm. This error is largely due to the signal to noise ratio in the complicated spectra, which under the present experimental conditions cannot be substantionally improved. Table 1 and 2 compare the values we obtain for the tensor elements with values of model compounds and bacteriorhodopsin published by Harbison et al. [18]. This comparison strongly suggest the presence of a 6-s-cis linkage in the chromophore of rhodopsin and the presence of a negative charge in the vicinity of carbon C-12.

The present results on two ^{13}C labeled rhodopsins prove that by means of high resolution solid state NMR spectroscopy very detailed information can be obtained about the structure of the chromophore and its interaction with the protein, as has been found before with bacteriorhodopsin. In addition to the isotropic chemical shift values, the three principal chemical shift tensor elements (σ_{11}, σ_{22} and σ_{33}) can be obtained as well. Such data provide information that is otherwise inaccessible. The σ_{11} value is an indicator for steric perturbation (γeffect), the σ_{22} value for charge density and σ_{33} may reflect isomeric configuration [17,18].

An unperturbed s-cis C-6 - C-7 single bond in rhodopsin

Valuable information on configuration and perturbation around carbon C-5 of the chromophore in rhodopsin can be deduced from comparison with reported values for the isotropic chemical shift and tensor elements for several retinal derivatives and bacteriorhodopsin at this position (Table 1). For carbon C-5 in rhodopsin the values for both the isotropic shift σ_i and the tensor elements σ_{11}, σ_{22} and σ_{33} are in close agreement with those found for triclinic retinoic acid. In the latter the conformation around the C-6 - C-7 single bond is a non planar 6-7 s-cis conformation, the thermodynamically more stable conformation, and the favored conformation of retinal in solution [18,32]. This means that the conformation of the 11-cis chromophore in rhodopsin is in the non planar unperturbed 6-7 s-cis form with no special interactions with the protein. This differs considerably from the situation in bacteriorhodopsin where the chromophore occurs in the planar 6-7 s-trans conformation (downfield shift of σ_{33}) and the C-5 carbon is perturbed by the close proximity of a negative charge from the peptide chain (downfield shift of σ_{22}).

Charge perturbation near carbon C-12 in rhodopsin

Due to the lack of appropriate crystalline 11-cis retinal derivatives much detailed information about position C-12 in a model system is not available. At this moment it is most appropriate to compare the values of the chemical shift tensor elements with those of carbon C-12 in bacteriorhodopsin$_{568}$, since there is no evidence that at this position special interactions occur with the protein moiety. The isotropic chemical shift value is also compared with those found for a 11-cis protonated Schiff base in solution (Table 2). The most pronounced difference occurs in the σ_{22}

Table.1. : The isotropic chemical shift and chemical shielding tensors of ^{13}C-5 Bovine Rhodopsin compared with those of several ^{13}C-5 retinoids having the 6 s-cis or 6 s-trans conformation.

COMPOUND	σ_1	σ_{11}	σ_{22}	σ_{33}	Reference
Retinoids with the 6-s-trans conformation					
Monoclinic All-trans Retinoic Acid	135.9	27	143	237	[17]
^{13}C-5 All-trans Retinylidene Bacterio-rhodopsin	144.8	27	170	237	[17]
Retinoids with the 6-s-cis conformation					
Triclinic All-trans Retinoic Acid	128.8	28	141	217	[17]
^{13}C-5 11-cis Retinylidene Bovine Rhodopsin	130.5	33	143	215	[this paper]

Table.2. : The isotropic chemical shift and chemical shielding tensors of ^{13}C-12 Bovine rhodopsin compared with those of several other retinoids.

COMPOUND	σ_1	σ_{11}	σ_{22}	σ_{33}	Reference
All-trans-Retinal	134	55	131	216	[18]
All-trans-Retinylidene unprotonated Schiff base	136	67	130	210	[18]
^{13}C-12 13-cis Retinylidene Bacterio-rhodopsin	124	32	134	205	[35]
^{13}C-12 all-trans Retinylidene Bacteriorhodopsin	134.3	56	130	211	[35]
^{13}C-12 11-cis Retinylidene Bovine Rhodopsin	133.5	41	149	209	[this paper]
11-cis Retinylidene Protonated Schiff base	129				[36]

value. The unperturbed state has σ_{22} values near 130 ppm irrespective of headgroup, but in rhodopsin it is shifted to 149 $\pm$ 6 ppm , i.e. a significant downfield shift of 19 $\pm$ 6 ppm. This downfield shift in σ_{22} might also explain why the isotropic shift in rhodopsin is shifted down-field relative to the unperturbed 11-cis retinylidene protonated Schiff base in solution. Harbison et al. [17] present several arguments that such a specific downfield shift in σ_{22} reflects accumulation of positive charge, which might be caused by interaction with a negative charge of the protein. Hence a downfield shift of the σ_{22} value in rhodopsin reflects the presence of a much larger positive charge on carbon C-12 then in bacteriorhodopsin. This will be due to the proximity of a negative charge in the protein near position C-12 in rhodopsin similarly as has been argued for carbon C-5 in bacteriorhodopsin [17].

The σ_{11} value of rhodopsin shows an upfield shift. As a matter of fact, the σ_{11} tensor value reflects steric interactions ,but it is not easy to find a satisfactorally explanation in this case, since the values for σ_{11} show considerable variance (Table 2) and reliable data for 11-cis retinylidene model compounds are still lacking.

Wavelength regulation in visual pigments

It is clear that the measurements on these two ^{13}C labelled rhodopsins show the power of contemporary solid state NMR techniques for the study of the interaction of the chromophore in the active site in the protein. The present study proves that the conformation of the C-6 - C-7 single bond is unperturbed non planar 6-7 s-cis. Although all details are not yet known, it also presents a clear indication for the presence of a negative charge in the neighbourhood of carbon C-12 in the chromophore of bovine rhodopsin. These data so far agree with theoretical models for wavelength regulation in rhodopsin [10]. However a more complete interpretation of the presented data has to be based on ^{13}C-NMR spectra of the other carbon atoms in the polyene chain of the chromophore. Studies on bovine rhodopsin labeled in other positions in retinal are in progress. Analysis by ^{13}C-CP-MAS NMR at low temperatures will further allow to investigate retinal conformation and retinal opsin interaction occurring after photon capture.

The difference in wavelength regulation in rhodopsin (6-s-cis, no charge near C-5, negative charge near C-12), and bacteriorhodopsin (6-s-trans, negative charge near C-5, no charge near C-12 [17], bivalent cations are implicated in wavelength regulation [33]) might indicate that there is no

close evolutionary relationship between these two classes of photosensory retinal based pigments: the visual pigment and the photoenergizing pigment. This tentative conclusion however awaits the analysis of cone pigments which show absorption maxima up into the spectral region of bacteriorhodopsin, and where monovalent anions may be involved in wavelength regulation [34].

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CHAPTER 9

SUMMARY AND DISCUSSION

In vertebrate retinas two types of light sensitive cells are found. The cone cells are responsible for colour vision and the rod cells mediate black and white vision. In this thesis the membrane of the light sensitive part, the outer segment, of the rod cell is the subject of the investigation described in this thesis.

The rod outer segment consists of a regular stack of membrane disks, surrounded by a plasma membrane. Compared to other biological membranes the composition of the disk membrane (photoreceptor membrane), which contains the light-sensitive intrinsic membrane protein rhodopsin, is relatively simple but rather uncommon. Over 90% of the membrane protein consists of rhodopsin, and more than 80% of the lipids is comprised of only three phospholipid classes (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine). About 55 - 60% of the phospholipid acyl chains are poly-unsaturated with an average number of 5.9 double bonds per phospholipid molecule. This high percentage of poly-unsaturation is exceptional. It seems inevitable that such a high degree of unsaturation affects the physico-chemical properties of this membrane.

The first event in vision is the capture of a light photon by rhodopsin. This will initiate a number of processes eventually leading to the hyperpolarization of the rod plasma membrane. A number of these processes occur within or on the surface of the photoreceptor membrane and will therefore be influenced by the general physico-chemical properties of this membrane. In this thesis the lipid phase-behaviour and lipid-rhodopsin interactions in this membrane are described, and the implication of the results for the physiology of vision is discussed.

In Chapter 2 a short description is given of the techniques employed for purification, identification and detection of the photoreceptor membrane lipids. A rapid and reliable method for the purification of isolated phospholipid classes according to headgroup is introduced, using analytical or preparative HPLC.

Chapters 3 and 4 describe the polymorphic phase-behaviour of photoreceptor membrane lipids in aqueous dispersions. It is shown that divalent cations, in particular calcium ions, can trigger the formation in these dispersions of non-bilayer lipid structures (hexagonal H_{II} phase and lipidic particles). It could also be demonstrated that this calcium-induced polymorphic phase behaviour was directly correlated to a 1:1 (molar)

interaction with phosphatidylserine. Maximal destabilization of the bilayer occurs at a molar calcium/phosphatidylserine ratio of 1:1 or more. Cholesterol, present in modest amounts in photoreceptor membranes, was found to lower the temperature of the bilayer to hexagonal H_{II} phase transition.

The purified phosphatidylethanolamine fraction showed formation of the hexagonal H_{II} phase in aqueous dispersions already at 0° C, as was expected for a poly-unsaturated phosphatidylethanolamine. Purified photoreceptor membrane phosphatidylserine spontaniously formed very small vesicles upon dispersion in calcium free buffer at neutral pH. Addition of calcium ions caused rapid fusion of the small vesicles to larger ones (> 200 nm), but in contrast to saturated or minimally unsaturated phosphatidylserine species no formation of strongly immobilized calcium-phosphatidylserine complex (cochleates) is observed.

Under all conditions, where the isolated photoreceptor membrane lipids show polymorphic phase behaviour, the photoreceptor membrane completely maintains the bilayer configuration. A bilayer stabilizing function for rhodopsin is postulated. In Chapter 5 this bilayer-stabilization potential of rhodopsin is further investigated. First of all, bilayer stabilization was still demonstrated in reconstituted membranes containing only rhodopsin and twice the natural amount of lipids. This result demonstrates that rhodopsin must be the bilayer stabilizing factor in photoreceptor membranes and that it has a high dynamic range. Further it is shown that the interaction between calcium and phosphatidylserine, which facilitates the polymorphic phase behaviour in the isolated lipids is not substantially infuenced by rhodopsin. In fact, in the presence of calcium the reconstituted membranes demonstrate fusogenic behaviour, whereby the rate of fusion seems to increase with increasing lipid/rhodopsin ratio. This indicates that, although the reconstituted membranes adopt an overall bilayer structure, transient non-bilayer structures need to be present to facilitate the vesicle fusion. The presence of rhodopsin apparently prevents that these local perturbations spread into phase changes. Finally, evidence is presented that the bilayer stabilizing property is related to an intact three dimensional structure of the protein: Non-bilayer structures can be induced in the photoreceptor membrane in the presence of calcium following thermal denaturation of rhodopsin or opsin.

In Chapter 6 the magnetic anisotropy of intact rod outer segments is used to obtain ^{31}P-NMR spectra of nearly perfectly oriented photoreceptor

membranes. ^{31}P-NMR spectra from such oriented membranes are quite different from randomly oriented membranes. In these spectra three different phospholipid pools can be detected and assigned to morphological substructures of the rod outer segment. The "oriented" spectra also show resolved peaks for the individual phospholipid classes. The intensity distribution of these peaks is in accordance with the phospholipid distribution in the photoreceptor membrane. The spectra facilitate the study of the dynamic behaviour of the phospholipids in these substructures. Our results gave no evidence for marked differences in T_1 relaxation time between the pools, or between the phospholipid classes. The absence of a peak around 7 ppm presents further proof for the complete absence of phospholipids organized in hexagonal H_{II} structures. From a detailed interpretation of the spectra, it seems unlikely that rhodopsin has an annulus of approximatily 25 constrained rigid lipids suggested in the literature.

Separate phospholipid pools as observed in the "oriented" spectra allow dynamic exchange studies using homonuclear saturation transfer. Such experiments are described in chapter 7 and indicate 1) free exchange of phospholipids within the disk flat and plasma membrane, 2) restricted exchange within the disk rim and between the disk rim and disk flat membrane. The restricted lateral phospholipid diffusion in the disk rim is probably caused by structural proteins in this membrane segment that are neccesary to maintain the high curvature important for the overal disk morphology.

In Chapter 8 an interesting side line is described which developed during the progress of this thesis. It demonstrates application of high resolution solid state ^{13}C-NMR to investigate the conformation of the retinal chromophore in rhodopsin and its interactions with the apo-protein, by using selectively labeled retinal. The obtained results indicate that in bovine rhodopsin the C-6 - C-7 single bond has the unperturbed cis conformation. A significant downshift in the value of the σ_{22} tensor element of the C-12 carbon could indicate the presence of a negative charge in the vicinity of this carbon atom. These findings so far support the current model for the wavelength regulation in bovine rhodopsin.

In the following sections the results described above according to chapter, will be placed in a broader context.

Possible relation with polymorphic phase behaviour

Dietary studies on animals indicate that the presence of poly-unsaturated fatty-acids are essential for proper vision. The function of this poly-unsaturation is not yet clear. It cannot be a matter of membrane fluidity, since this does not markedly change when the number of unsaturated bonds per acyl chain exceeds two. In the following discussion evidence is presented that poly-unsaturation in the photoreceptor membrane is relevant for its function as a permeability barrier.

The most important function of a biological membrane is to generate a physical structure which acts as a selective permeability barrier separating two aqueous compartments of specific composition. In the case of the rod outer segment, the photoreceptor membrane is responsible for the special, almost crystalline, architecture of this cell organel and it separates the intra-diskal space from the cytosol.

The mechanisms which facilitate selective passive transport e.g. of polar solutes accros a lipid bilayer, are still not well understood. The potential of certain lipids occurring in biological membranes, like unsaturated phosphatidylethanolamine, to adopt non-bilayer structures such as "inverted micelles", generates attractive models to explain this selective permeability. Several experimental results in literature indeed indicate that the transport of divalent cations accros lipid bilayer membranes is facilitated by the presence of non-bilayer structures. This might be relevant to the physiology of the rod outer segment. There is evidence that a transient increase in calcium permeability of the photoreceptor disk membrane occurs after light excitation. In addition the photoreceptor membrane lipid matrix contains a high percentage of poly-unsaturated phosphatidylethanolamine. Therefore transient formation of inverted micelles in the photoreceptor membrane could play a role in the permeability increase for calcium ions. However, hitherto no study on the phase behaviour of lipids of the photoreceptor membrane was performed.

The results presented in this thesis clearly show that the lipid matrix present in photoreceptor membranes indeed is capable of adopting non-bilayer structures under conditions present in the "in vivo" rod outer segment. The driving force behind the formation of these structures is a combination of: 1) The preference of the poly-unsaturated phosphatidylethanolamine fraction to

organize in the hexagonal H_{II} phase, and 2) The interaction of calcium ions with phosphatidylserine, as a 1:1 complex. The modest amount of cholesterol, present in the photoreceptor membrane lipid matrix, effectuates a decrease in transition temperature so that non-bilayer structures is already complete under physiological conditions.

Such data support the idea that non-bilayer structures might be relevant for the function of the photoreceptor membrane. However, the intact photoreceptor membrane does not give any evidence for the presence of non-bilayer structures under physiological conditions. Rather, all evidence indicates that the presence of rhodopsin completely suppresses the tendency of the photoreceptor lipids to form non-bilayer structures. Further investigation revealed that this bilayer stabilization was not due to interference of rhodopsin with the interaction between calcium and phosphatidylserine. In fact an intact protein structure seems to be essential, since upon thermal denaturation of rhodopsin and opsin non-bilayer structures can also be induced in the photoreceptor membrane by calcium ions. Analysis of reconstituted membranes, consisting of photoreceptor membrane lipids and rhodopsin but with a lipid/rhodopsin ratio twice that of the natural membrane, demonstrated that even under these circumstances the overall bilayer organization was maintained. However, the resulting vesicles easily underwent fusion in the presence of calcium, indicating that these membranes allow transient bilayer perturbations necessary to initiate the fusion event.

Membrane permeability and fluidity

In addition to the observed bilayer stabilization a second aspect of protein-lipid interaction might be relevant in this context. It is well documented that the permeability of a membrane for polar solutes, depends on the sealing properties of the lipids at the membrane protein-lipid interface. Here both phosphatidylethanolamine and phosphatidylserine could be implicated.

Poly-unsaturated phosphatidylethanolamine:

As discussed above the presence of transient non-bilayer structures in the photoreceptor membrane cannot yet be excluded. However, direct evidence for non-bilayer structures in the photoreceptor membrane under physiological conditions is lacking. Therefore, the high degree of poly-unsaturation of the phosphatidylethanolamine species is not primarily meant to allow easy induction of non-bilayer structures. Another explanation might be that the presence of large amounts of poly-unsaturated phosphatidylethanolamine is

required for proper "sealing" of the photoreceptor membrane.

From a wealth of studies on lipid polymorphism, the picture has emerged that a close link exists between the overall shape of a phospholipid molecule and its phase behaviour in aqueous dispersions (see Chapter 1). Cone-shaped phospholipids like poly-unsaturated phosphatidylethanolamine prefer the hexagonal H_{II} phase. In a lipid mixture the tendency of cone-shaped phospholipids to form a hexagonal H_{II} phase can be counteracted by lipids having an inverse cone-shape (e.g. lyso-phospholipids).

According to Israelichvili [(1977) Biochim. Biophys. Acta 469, 221-225] the form of the outer surface of the intrinsic part of the membrane proteins has to match the surrounding lipids in order to produce a membrane with good sealing properities. Cone-shaped phospholipids like poly-unsaturated phosphatidylethanolamine in the photoreceptor membrane might therefore be required in the photoreceptor membrane to match an inverted cone-shape of the hydrophobic interface of rhodopsin. This would immediately explain the strong bilayer stabilizing effect of rhodopsin on the lipid matrix and the loss of this stabilization upon thermal denaturation. Good sealing properties are probably essential for a membrane where the regulation of ion-fluxes is an important functional aspect.

The existence of ultra-long poly-unsaturated fatty acyl chains (28 to 32 carbon atoms) in the photoreceptor membrane in appreciable amounts might be related with the sealing qualities of this membrane as well.

Poly-unsaturated phosphatidylserine:

Saturated and mono-unsaturated phosphatidylserine are known to form crystalline complexes with calcium in the form of concentric rigid bilayers. As was shown, the behaviour of the isolated poly-unsaturated photoreceptor phosphatidylserine in the presence of calcium is quite different from that observed for the more saturated species. It does not markedly rigidify but largely maintains a liquid-crystalline structure. Also in mixed lipid membranes, like the photoreceptor membrane, poly-unsaturated phosphatidylserine behaves different from more saturated phosphatidylserine species in that addition of calcium ions does not lead to lateral separation of the phosphatidylserine molecules. In view of the fact that in several parts of the rod outer segment high concentrations of calcium (up to 100 mM) and magnesium (2-4 mM) are present. Therefore a possible function for the poly-unsaturation of phosphatidylserine might be related to the maintainance of a fluid photoreceptor membrane under ion stress. Furthermore the unsaturation of

phosphatidylserine prevents its lateral separation from the other lipids in the natural membrane under physiological conditions, which could seriously perturb the membrane structure and affect its permeability properties.

CONCLUSION

The photoreceptor membrane seems to be in a sort meta-stable state in which two forces counteract. One drives towards non-bilayer structures and the other one surpresses this tendency. Under normal physiological conditions the latter force dominates. We propose that :

1. In the photoreceptor membrane a high content of phosphatidylethanolamine is required to give good sealing with rhodopsin.
2. poly-unsaturation of phosphatidylethanolamine and phosphatidylserine is required to maintain high fluidity in spite of strong interactions with proteins or bivalent cations.
3. Diffusion barrier property of the disk rim guarantees optimal positioning of rhodopsin and possible recognition sites for signal transducing protein attachment.

In de retina van vertebraten bevinden zich twee soorten licht gevoelige cellen. De kegeltjes cellen die verantwoordelijk zijn voor het kleurzien en de staafjes cellen waarmee slechts zwart-wit beelden verkregen worden. De laatste echter maken, door hun relatief veel hogere licht gevoeligheid, het zien mogelijk onder omstandigheden met zeer weinig licht. Het proefschrift beschrijft een fysisch-chemische en biochemische studie aan het licht gevoelige "fotoreceptor membraan" dat geisoleerd kan worden uit deze staafjes cellen.

De belangrijkste functies van een biologisch membraan zijn: 1. Het is een selectieve barriere tussen cel compartimenten met een verschillende samenstelling, en tussen de cel als geheel en zijn omgeving. 2. Het is een structureel element dat de vormgeving van de cel bepaald. 3. Het is een matrix voor veel membraan eiwitten. Ook het fotoreceptor membraan vervult deze functies in de staafjes cel. Het geeft vorm aan de bijna cristallijne stapeling van de membraan zakjes "disks", scheidt de de sterk afwijkende inhoud van deze disks van het cel cytoplasma en het bevat een hoog percentage membraan eiwit. Specifiek voor het fotoreceptor membraan is haar functie bij het zien.

Het fotoreceptor membraan heeft een relatief simpele, maar zeer bijzondere,samenstelling. Meer dan 90% van het membraan eiwit bestaat uit het licht gevoelige intrinsieke membraan eiwit rhodopsine, en meer dan 80% van de lipiden bestaat uit slechts 3 klassen fosfolipiden. Fosfatydilethanolamine, fosfatydilcholine en fosfatydilserine. Een zeer hoog percentage, 55%-60% van de vetzuur ketens van deze fosfolipiden is meervoudig onverzadigd, met een gemiddelde van 5.9 dubbele banden per acyl keten. Deze hoge mate van onverzadigdheid is uniek voor een biologisch membraan en moet invloed hebben op de fysisch- chemische eigenschappen van het fotoreceptor membraan. Dieet studies met dieren hebben eenduidig aangetoond dat een gebrek aan hoogoverzadigde vetzuren leidt tot degeneratie van het fotoreceptor membraan en verlies van gezichts vermogen.

Een aantal processen betrokken bij het omzetten van de lichtprikkel in een electrische prikkel in de staafjes cellen vindt plaats in of op het fotoreceptor membraan, en zal als zodanig afhankelijk zijn van de fysische eigenschappen van dit membraan, zoals deze zich manifesteren in de vloeibaarheid, het lipid fase gedrag en de lipid-eiwit interacties. Deze

eigenschappen zijn in het fotoreceptor membraan tot nu toe nog niet goed onderzocht.

In het hier beschreven onderzoek is met behulp van twee fysisch-chemische technieken : kern magnetische resonantie (NMR) en electronen microscopie (EM) het fasegedrag van de lipiden uit dit membraan onderzocht.

In hoofdstuk 2 wordt een korte beschrijving gegeven van de technieken die in het onderzoek zijn gebruikt voor de zuivering en identificatie van de fotoreceptor membraan lipiden. Een snelle en betrouwbare methode voor de scheiding van de verschillende fosfolipid klassen, zowel analytisch als preperatief, wordt geintroduceerd.

De hoofdstukken 3 en 4 beschrijven het polymorphe fase gedrag van de fotoreceptor membraan lipiden in waterige suspensies. Er wordt aangetoond : 1. De fotoreceptor membraan lipiden, in afwezigheid van rhodopsine, vrij gemakkelijk overgaan tot vorming van niet bilaag structuren zoals de "hexagonale H_{II} phase" en "lipid partikels". Deze niet bilaag structuren komen echter alleen voor in aanwezigheid van divalente cationen en temperaturen boven 20 ^{o}C. 2. Dit polymorf fasegedrag wordt geinduceerd door een interactie tussen het divalente cation Ca^{2+} en het fosfolipid fosfatydilserine. 3. Gevonden werd dat de 12 mol% cholesterol in het fotoreceptor membraan een duidelijk stimulerende werking heeft op de vorming van deze niet bilaag structuren, vergelijkbaar met een temperatuurs verhoging van 20 ^{o}C. 4. De vorming van niet bilaag structuren optreedt onder physiologische omstandigheden van temperatuur, ion sterkte en pH. In het native fotoreceptor membraan blijken de lipiden zich echter onder alle bovengenoemde omstandigheden exclusief in een bilaag te organizeren. Ook belichting heeft hierop geen waarneembaar effect. De bilaag organisatie wordt zelfs gehandhaafd bij temperaturen tot minimaal 50 ^{o}C.

Het rhodopsine is blijkbaar in staat de bilaag organisatie van de fotoreceptor membraan lipiden te stabiliseren. Een aantal aspecten van dit fenomeen is nader onderzocht. Uit de resultaten blijkt dat deze bilaag stabiliserende werking van het rhodopsine : 1. Niet wordt veroorzaakt door een beinvloeding van de calcium/fosfatydilserine binding door rhodopsine. 2. Een grote moleculaire efficientie heeft. Zij strekt zich minstens uit tot 150 lipiden per rhodopsine, fuim tweemaal de native rhodopsine/lipid ratio (1:65). 3. Er een directe correlatie is tussen het opkomen van polymorf fase gedrag bij verhoogde temperaturen (>50 ^{o}C), en de denaturatie van de tertiare structuur van het rhodopsine ten gevolge van deze verhoogde temperaturen. 4.

Er geen directe correlatie is tussen de aanwezigheid van niet bilaag structuren en licht, de stimulus van het fotoreceptor membraan.

Alhoewel de fosfolipiden uit het fotoreceptor membraan onder fysiologische omstandigheden in afwezigheid van rhodopsine (met een intacte tertiaire structuur) polymorf fasegedrag vertonen, kon niet worden aangetoond dat deze structuren ook optreden in het native membraan onder fysiologisch relevante condities. Voor de fysiologie van "het zien" betekenen deze resultaten dat er geen direct eenduidige uitspraak kon worden gedaan of polymorf fasegedrag van lipiden als zodanig direct betrokken is bij de processen die in het staafje de licht prikkel omzetten in een electrische prikkel. Dit impliceert dat de functie van de meervoudig onverzadigde fosfolipiden in het fotoreceptor membraan waarschijnlijk niet gecorreleerd is aan het afwijkend phasegedrag van deze lipiden. Echter de gebruikte technieken zijn niet in staat zeer kleine percentages niet bilaag structuur te detecteren zodat niet uitgesloten kan worden dat niet bilaag structuren toch optreden op micro schaal in het native membraan, en daarbij op een moleculair niveau in een micro omgeving toch betrokken zijn bij boven genoemde fysiologische processen in de staafjes cel.

De functie van deze hoogonverzadigde fosfolipiden lijkt meer verbonden te zijn met de barriere functie van het fotoreceptor membraan. Argumenten hiervoor worden aangedragen.

In hoofdstuk 6 de magnetische anisotropie van de intacte staafjes cellen is gebruikt om ^{31}P-NMR spectra te verkrijgen van een bijna perfect georienteerd membraan. De vorm van deze spectra wijkt sterk af van die gevonden voor een niet georienteerd membraan. In de georienteerde spectra worden verschillende lipid pools gevonden, die gecorrelleerd kunnen worden aan de morphologie van de staafjes cel. In hoofdstuk 7 wordt deze eigenschap gebruikt om de uitwisseling van lipid materiaal tussen de verschillende morphologische subunits te onderzoeken. Aangetoond wordt dat er in de staafjes cel units zijn waartussen slechts gelimiteerde diffusie van lipid materiaal plaats vindt.

In het proefschrift wordt ook, als een interessante zijlijn naast het onderzoek van het lipid fase gedrag, een ^{13}C solid state NMR studie beschreven naar de configuratie van de chromofoor in rhodopsine. De resultaten van deze studie, beschreven in hoofdstuk 8, ondersteunen het in de literatuur voorgestelde model voor de vorm van de chromofore pocket.

Graag wil ik eenieder bedanken die aan het tot stand komen van dit proefschrift heeft bijgedragen. Een aantal van hen wil ik met name noemen:

Diny van Groningen-Luyben, Petra Bovee-Geurts en Jenny van Oostrum voor hun vakkundige hulp bij het uitvoeren van een groot aantal experimenten.

De mede promovendi Ad Timmers en John Schalken voor hun adviezen en kritische inbreng tijdens de woensdag avond discussies.

Jan Labro en Wim Somers die in het kader van hun doctoraalstage een bijdrage aan het onderzoek hebben geleverd.

Prof. Edward Dratz for his stimulating contribution and expert advise, which resulted in a joint publication (hoofdstuk 6).

Je voudrais remercier Dr J. Olive pour son travaille excellent de microscopie electronique.

Prof Wibren Veeman en Dr Arno Kentgens voor hun onmisbare bijdrage aan de vaste stof NMR experimenten, die beschreven staan in hoofdstuk 8.

Prof J. Lugtenburg voor het bereidwillig beschikbaar stellen van de gelabelde retinals.

Buiten de laboratorium omgeving alle vrienden en vriendinnen voor hun belangstelling in mijn werkzaamheden, ook al ontging hun vaak de diepere betekenis achter het snijwerk in al die runder ogen.

Tenslotte, maar niet op de laatste plaats, mijn vader en moeder dat zij mij de mogelijkheid geboden hebben om te studeren en hun altijd bemoedigende belangstelling.

Leo Mollevanger werd geboren op 21 januari 1956 te Nijmegen. Van 1960 tot 1962 bezocht hij de kleuterschool "Pinkeltje" te Hattem. Hierna volgde hij in dezelfde plaats tot 1968 lager onderwijs aan de "St. Andreasschool". In augustus 1968 werd begonnen aan de middelbare schoolopleiding aan het Jeroen Bosch College te 's-Hertogenbosch. Het Atheneum-B diploma werd behaald in 1974. Direct hierop aansluitend werd een aanvang gemaakt met de studie scheikunde aan de Katholieke Universiteit Nijmegen. In 1977 werd het kandidaats examen S_2 behaald. Het doctoraal examen werd afgelegd in 1981, met als hoofdrichtingen Organische Chemie (Prof. Dr. G.I. Tesser) en Biofysische Chemie (Prof. Dr. C.W. Hilbers). Van 1981 tot en met 1985 was hij werkzaam op het Laboratorium voor Biochemie van de Medische Faculteit van de Katholieke Universiteit Nijmegen, in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO). Gedurende deze periode werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof. Dr. F.J.M. Daemen, Prof. Dr. C.W. Hilbers, en Dr. W.J. de Grip. Op het ogenblik is hij werkzaam als applicatie scientist voor magnetische resonantie spectroscopie bij de Medical Systems Divisie van Philips in Best.

STELLINGEN

I

De conclusie van Albert en Yeagle, dat 25% van de fosfolipiden in het fotoreceptormembraan sterk geimmobiliseerd is door rhodopsine, is niet aannemenlijk.

Albert, A.D. and Yeagle, P.L. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 7188-7191

II

Het verschil in fasegedrag van fosfolipiden kan slechts een kwestie zijn van 1+ of 2+.

De Grip, W.J., Drenthe, E.H.S., Van Echteld, C.J.A., De Kruijff, B. and Verkleij, A.J. (1979) Biochim. Biophys. Acta 558, 330-337

Deese, A.J., Dratz, E.A., and Brown, M.F. (1981) FEBS Lett. 124, 93-99

III

Het getuigd van een grote mate van academische "vrijheidsgraad" om bij een Moleculair Dynamische studie aan het eiwit Crambine, uit de vier theoretisch mogelijke homologen van dit eiwit, juist te kiezen voor de niet voorkomende homoloog.

Teeter, M. in "Proceedings of a workshop on molecular dynamics and protein structure" (1981) Ed. J. Hermans, University of North Carolina.

Teeter, M. M., Mazer, J.A. and L'Italien, J.J. (1981) Biochemistry 20, 5437-5443

IV

Het is niet mogelijk om de ISIS techniek, voor volume selective magnetische resonantie spectroscopie, toe te passen in combinatie met oppervlakte spoelen.

Ordidge, R.J., Connelly, A. and Lohman, J.A.B. (1986) J. Magn. Reson. 66, 283-294

V

Het gebruik van de Western-Blotting methode, voor een quantitatieve bepaling van de hoeveelheid rhodopsine in normale en RD mutante muizen, behoeft ten minste dat wordt aangetoond dat deze techniek geschikt is voor quantitatieve analyses.

Takemoto, D.J., Hansen, J. and Takemoto, L.J. (1985) Biochem. Biophys. Res. Comm. 132, 804-810

VI

Als bij een studie aan maligne humane tumoren met behulp van hoge resolutie magnetische resonantie spectroscopie (MRS), er slechts bij 2.7% van de onderzochte tumoren een significant verschil in de spectra wordt gevonden kan moeilijk gesproken worden van een classificatie van humane tumoren met behulp van MRS.

Mountford, C.E., May, G.L. Williams, P.G., Tattersall, M.H.N., Russell, P., Saunders, J.K., Holmes, K.T., Fox, R.M., Barr, J.R. and Smith, I.C.P. (1986) The Lancet 8482, 651-653

VII

Het heffen van een auteurs-rechterlijke bijdrage op onbespeelde audio en video banden geeft het "illegaal copieren" een legale bases.

VIII

De huidige toepassing van de wet militaire dienstplicht is in strijd met artikel I van de grondwet.

Nijmegen, 30 januari 1987

Leo Mollevanger